Using Drosophila to study the contribution of human kinases to tau toxicity

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Abstract

The major neuropathological feature associated with tauopathies is the aggregation of the microtubule associated protein tau within neurons. Aberrant phosphorylation events on tau contribute to the aggregation during disease. The kinases and the phosphorylation sites on tau responsible for the generation of pathogenic tau forms are still unclear. My thesis proposes to establish a *Drosophila* model to investigate the human kinases and phosphorylation sites responsible for the generation of human tau toxicity. Expression of human tau in the photoreceptors of transgenic *Drosophila* leads to a degenerative phenotype proportional to the transcriptional level of the transgene. Since the genomic position of the transgene influences its expression, I used a novel method of transgenesis to insert the human tau transgene in a reproducible position within the fly genome. Using this system, tau isoforms differing for the presence of two N-terminal domains were shown to generate the same level of toxicity in the fly eye. Moreover, in contrast with other studies, the tauopathy-associated mutation R406W on human tau did not cause an enhancement of tau-mediated degeneration of the fly eye. Selected human kinases relevant to tau pathology were tested for their ability to enhance tau toxicity in flies. Only human GSK3β showed a robust enhancement of tau-mediated eye degeneration. For the first time I showed that a human kinase, GSK3β, could specifically phosphorylate human tau in *Drosophila* on sites not targeted by endogenous fly kinases. In particular, tau phosphorylation of T181, S396 and S404 was found to significantly increase upon GSK3β expression. Site-directed mutagenesis on tau suggested that S404 might play a central role in mediating human tau toxicity in the fly eye. Finally this thesis demonstrates that the *Drosophila* model of tauopathy here created can be used to assess the importance of human kinases and of single phosphorylated sites in the generation of tau toxicity.

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Abbreviations

Aβ	Amyloid beta
AD	Alzheimer's disease
AGD	Argyrophilic grains disease
AMP	Adenosine monophosphate
APP	Amyloid precursor protein
APS	Ammonium persulphate
bp	Base pair
C-terminal	Carboxy-terminal
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic AMP
CBD	Corticobasal degeneration
CD8	Cluster of differentiation 8
Cdk5	Cyclin-dependent kinase 5
cDNA	Complementary DNA
CK1	Casein kinase 1
CNS	Central nervous system
dH_2O	Distilled water
dNTPs	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
DNTC	Diffuse neurofibrillary tangle dementia with calcifications
DS	Down syndrome
DYRK1A	Dual-specificity tyrosine-phosphorylation regulated kinase 1A
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethylene diamine tetra-acetic acid
FTLDs	Frontotemporal lobar degenerations
FTLDs-tau	Frontotemporal lobar degenerations associated with tau
FTDP-17	Frontotemporal dementia with Parkinsonism linked to
FUS	Tumour-associated protein fused in sarcoma
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase 3β
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kDa	Kilo dalton

LB	Luria Bertani
MAP	Microtubule associated protein
MAPK	Mitogen activated protein kinase
MARK	Microtubule affinity regulating kinases
mRNA	Messenger RNA
MSC	Sporadic multisystem tauopathy with globular inclusions
N-terminal	Amino-terminal
NFTs	Neurofibrillary tangles
NS	Non-significant
PAR-1	Partitioning defective-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHF	Paired helical filaments
PiD	Pick's disease
PKA	Cyclic AMP-dependent protein kinase
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B
PSP	Supranuclear palsy
RNA	Ribonucleic acid
S2	Schneider 2
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SEM	Standard error of the mean
TAE	Tris acetate EDTA
TDP-43	Transactive response DNA binding protein of 43 kDa
TE	Tris EDTA
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
UAS	Upstream activation sequence
v/v	Volume by volume
VaD	Vascular dementia
w/v	Weight by volume

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CHAPTER 1
Introduction

1.1 Dementia

Dementia is a progressive terminal condition affecting the elderly that damages memory, thinking, behaviour as well as the ability to perform everyday activities. After the age of 65, the likelihood of developing dementia doubles every five years. Currently 36 million people are affected by dementia worldwide and this figure is steadily increasing. The number of people affected by dementia in Europe and USA is estimated to be 9.95 million and 4.38 million respectively (Wimo & Prince, 2010). The assistance and medical care for dementia patients represent a true economic burden for governments worldwide, in particular in Europe and USA. In 2050, the incidence of senile dementia and its related costs are expected to triple (Thies et al, 2011).

Dementia is a collection of disorders characterised by a progressive cognitive decline causing functional impairments and generally affecting the ageing population over 65 years old. The diagnosis of dementia is formulated on the basis of several criteria published in the Diagnostic and Statistical Manual of Mental Disorders (1994). According to the manual, the symptoms of dementia include a progressive decline in memory and cognitive abilities severe enough to interfere with daily life. The cognitive abilities usually affected include generating coherent speech, understanding spoken or written language, recognising objects, executing motor activities, thinking abstractly, making judgments, and carrying out complex tasks. Dementia can be caused by various diseases and conditions, among which the neurodegenerative pathologies are characterised by the progressive and irreversible deterioration of brain cells and their interconnections (Dickson et al, 2011; Josephs et al, 2011). There are four major types of neurodegenerative dementia conditions associated with distinct symptomatic patterns and different brain abnormalities, including Alzheimer's disease (AD), vascular dementia (VaD), Lewy body dementia, and frontotemporal lobar degenerations (FTLDs). Ultimately, the gradual worsening course of neurodegeneration leads to death caused by neuronal loss.

AD is the most common type of dementia accounting for an estimated 60% to 80% of cases (Thies et al, 2011). The most common pattern of disease begins with a slow progressive decline in memory and, in later stages, with confusion, disorientation,

impaired communication and loss of motor function. The neuropathological hallmark of AD is the deposition of amyloid plaques and neurofibrillary tangles (NFTs), composed of β-amyloid (Aβ) and tau proteins, respectively (Braak & Braak, 1996; St George-Hyslop, 2000). Vascular dementia is considered the second most common type of dementia (Thies et al, 2011). During disease, a series of small strokes block the arteries causing a reduction of blood flow to parts of the brain. Symptoms often overlap with those of AD, although memory may not be as seriously affected. Importantly, vascular disease may predispose some individuals to acquire AD, or may exacerbate its clinical course (Iadecola, 2010). In dementia with Lewy bodies, the pattern of neurological decline may be similar to AD, including memory and judgment impairments and behavioural changes. However, the progression is more rapid and also includes visual hallucinations, muscle rigidity and tremors. The molecular hallmark of this type of dementia is the pathogenic deposition of the α-synuclein protein leading to the formation of Lewy bodies inside neurons (Sonnen et al, 2010). FTLDs are characterised by a slow progression of degeneration affecting mainly the frontal and temporal lobes of the brain. Typical symptoms include changes in personality and behaviour as well as difficulty with language. The neuropathological features of FTLDs are associated with three proteins that independently contribute to disease: tau, the transactive response DNA binding protein of 43 kDa (TDP-43) and the tumour-associated protein fused in sarcoma (FUS) (Hutton et al, 1998; Kwiatkowski et al, 2009; Neumann et al, 2006). The FTLDs associated with tau (FTLDs-tau) consist of a group of diseases in which the major pathogenic mechanism is the precipitation of highly phosphorylated tau aggregates in the brain leading to cell death (Dickson et al, 2011; Josephs et al, 2011).
Increasing evidence from long-term studies indicates that many people with dementia have brain abnormalities associated with more than one type of dementia. Mixed dementia is characterised by the hallmarks of AD and another type of dementia, most commonly vascular dementia or dementia with Lewy bodies (Table 1.1) (Jellinger & Attems, 2007).

No treatment is available to prevent or slow the neurodegeneration occurring during dementia. Current drugs temporarily slow the worsening of symptoms, however they are effective for only about half of the patients. Therefore, understanding the underlying biology of neurodegeneration that occurs during dementia is crucial in order to identify treatment strategies.

	Prevalence	Clinical symptoms	Neuropathological features	Aggregated protein
Alzheimer's disease	60-80%	Memory loss, confusion, behavioral changes, language impairment	Deposition of amyloid plaques and neurofibrillary tangles	Aβ and tau proteins
Vascular dementia	17%	Mild memory loss, confusion, behavioral changes, language impairment	Small strokes in the brain arteries	-
Lewy body dementia	10%	Memory loss, confusion, behavioral changes, language impairment, visual hallucinations, muscle tremors	Deposition of Lewy bodies	α-synuclein
FTLDs-tau	5%	Psychosis, severe behavior and language disturbances, cognitive dysfunction, motor symptoms	Deposition of tau aggregates	Tau protein
Mixed dementias	5%	Alzheimer's disease with Lewy bodies or vascular dementia	Deposition of tau aggregates and Lewy bodies or strokes in the brain arteries	Tau protein only or with α-synuclein

Table 1.1 Neurodegenerative dementias. The main types of dementia with neurodegenerative pathologies are listed together with their prevalence, clinical symptoms, neuropathological features and types of protein aggregates present.

1.2 Protein aggregation and neurodegeneration

Although dementia disorders affect different brain regions and lead to different symptomatic patterns, the neurodegenerative process presents common features (Jellinger, 2011). The cellular and molecular mechanisms in common between the dementia disorders are the toxic protein aggregation and inclusion body formation in selected areas of the brain. The pathologies characterised by protein aggregation and accumulation are also referred to as proteinopathies (Golde & Miller, 2009; McMillan & Leverenz, 2010). Accumulation of misfolded, aggregated proteins in the brain triggers a complex series of events that result in neuronal degeneration and death (Ross & Poirier, 2004). Although protein inclusions have been shown to contribute to neurodegeneration in dementia, the mechanisms leading to neuronal toxicity are still unclear. Proteins widely known to aggregate and accumulate in the brain of patients affected by dementia are Aβ, tau and α-synuclein. During AD, the deposition of the Aβ protein and tau has been demonstrated as key pathogenic triggers for neurodegeneration (Hardy & Allsop, 1991). Moreover, misfolding and subsequent aggregation of α-synuclein play a central role in the pathogenesis of Lewy body dementia and other synucleinopathies (Uversky, 2007). Finally, the aggregation and precipitation of tau alone has been shown to be the major pathogenic event in the neurodegenerative process of FTLDs-tau (Lee et al, 2001).

Proteins involved in neurodegenerative processes are natively unfolded, however, during disease they aggregate and precipitate. The protein deposits found in dementia brains are characterised by the same abnormal structural conformation including amyloid fibrils (Ross & Poirier, 2004). Amyloid fibrils consist of insoluble filamentous aggregates containing misfolded protein with β-sheet formation (Sunde & Blake, 1998). The most extensively characterised amyloid fibril is the one formed by the Aβ peptide implicated in AD (Török et al, 2002). During aggregation, tau often adopts higher structures, in particular β-structures, although it normally exists as an unstructured protein due to a high percentage of hydrophilic residues (Jeganathan et al, 2008). Also, similar fibrils are formed by α-synuclein (Der-Sarkissian et al, 2003). The detailed structure of amyloid fibrils and their assembly are still unknown, however it has been

noticed that the structures of different kinds of disease-related protein aggregates share considerable similarities.

Protein aggregation is a complex process involving the formation of oligomeric intermediates that go on to form aggregated fibers. In many dementia disorders, aggregation intermediates have also been observed to confer toxicity in neurons. These small aggregated structures are known as oligomers and they are usually highly stable, diffusible and non-fibrillar (Gadad et al, 2011). Most commonly they initiate the impairment of neuronal transmission and functioning at the synaptic site (Schulz-Schaeffer, 2010; Takahashi et al, 2010). It is thought that the toxic oligomers are sequestered in larger polymers leading to the formation of inclusion bodies and extracellular deposits (Lansbury & Lashuel, 2006). However, the role that the oligomeric intermediates and the large insoluble deposits play in synaptic and mitochondrial dysfunction, neuronal apoptosis and cell death is still debated (Jellinger, 2011). These inclusions and aggregates result from a molecular cascade of events probably caused by an imbalance between protein synthesis, aggregation and clearance (Gadad et al, 2011). The pathogenic mechanism of the intermediate aggregation is still unclear, however, aggregated soluble non-fibrillar intermediates with similar size and morphology exist in Aβ, tau and α-synuclein pathologies.

The initiation of protein misfolding in a cell may be a stochastic event, however, several factors may facilitate protein aggregation during dementia. Recent studies suggest that propagation of misfolded protein might occur in neurodegenerative diseases, including AD, Lewy body dementia and FTLDs-tau and this might contribute to the spreading and progression of the disease (Frost & Diamond, 2010; Jucker & Walker, 2011; Lee et al, 2010). The formation of protein aggregates catalyses the structural conversion of the normally folded proteins into additional aggregates via a process of seeded polymerisation. For instance, extracellular Aβ and α-synuclein oligomers can induce intracellular aggregation of the same proteins (Danzer et al, 2009; Rosen et al, 2011). Moreover, also tau aggregates can induce normal tau to aggregate (Guo & Lee, 2011). Furthermore, post-translational modification of dementia proteins may facilitate their aggregation and precipitation. Oxidative stress causing protein modification is increased with age, one of the major risk factors for dementia (Sultana & Butterfield, 2010). For example, aggregation of α-synuclein has been shown to be facilitated by oxidative

modifications (Giasson et al, 2000). Another important post-translational modification promoting protein aggregation is phosphorylation. In Lewy body dementia, α-synuclein has been found to be extensively phosphorylated on S129 and this phosphorylation event appears to accelerate the formation of inclusions during disease (Iwatsubo et al, 1996; Spillantini et al, 1997). Aberrant phosphorylation events are also implicated in AD and FTLDs-tau leading to tau hyperphosphorylation and aggregation in NFTs or other types of tau deposits (Lee et al, 2001). The pathological cascade of protein aggregation can also be triggered by mutations in a number of genes that increase the likelihood of accumulation of protein aggregates (Bertram & Tanzi, 2005). In AD, many genetic mutations have been identified to increase Aβ aggregation into amyloid plaques (Hashimoto et al, 2000). Moreover, several mutations in the tau gene have been associated with specific forms of FTLDs-tau and increase its likelihood to aggregate into filaments (Spillantini et al, 1998; van Swieten & Spillantini, 2007).

Protein misfolding and aggregation represent important aspects of the neurodegenerative process that occur during the pathogenesis of dementia. Investigating common pathological mechanisms that underlie neurodegeneration in dementia could lead to the discovery of therapeutic targets common to several dementia conditions. The aggregation of the tau protein is a neuropathological feature of great importance in the pathogenic mechanisms underlying AD and FTLDs-tau. For this reason the subset of dementia disorders characterised by the deposition of tau aggregates are also known as tauopathies. Tau is a microtubule-associated protein (MAP) whose function is regulated by several post-translational modifications, including phosphorylation. However, in tauopathy brain aberrant phosphorylation events contribute to the formation of tau deposits in neurons and glia that lead to neurodegeneration and ultimately cell death. The full inventory of kinases involved in the hyperphosphorylation of tau, and their pathogenic sites of phosphorylation, is still unknown. In recent years, investigations have focused on identifying the pathogenic kinases involved in tau hyperphosphorylation, since these enzymes could be targets for new therapies that reduce tau pathology and neuronal loss occurring in AD and FTLDs-tau (Lee et al, 2011).

1.3 The structure of tau

Tau proteins belong to the MAP family that binds to and stabilises microtubules. It was first described by Weingarten et al (1975) as a heat-stable protein essential for microtubule assembly. In porcine brain, tau was found to be present in association with tubulin and *in vitro* experiments showed that tau is necessary for tubulin assembly into microtubules (Weingarten et al, 1975).

Tau is encoded by a single-copy gene located on the long arm of chromosome 17 (17q21.1) in humans (Goedert et al, 1988; Himmler, 1989; Neve et al, 1986). The tau primary transcript contains sixteen exons, the majority of which are constitutively expressed, while exons 2, 3 and 10 are alternatively spliced (Figure 1.1). In adult human brain, alternative splicing generates six tau isoforms ranging from 352 to 441 amino acids in length. Exons 2 and 3 encode two amino (N)-terminal inserts in tau, each characterised by a highly acidic twenty-nine amino acid sequence. Alternative splicing generates tau isoforms carrying either no N-terminal insert (0N, exons 2 and 3 absent), one N-terminal insert (1N, exon 2 present) or two N-terminal inserts (2N, exons 2 and 3 present). Exons 9, 10, 11 and 12 are responsible for encoding four repeated regions in the carboxy (C)-terminal half of tau, characterised by conserved repeats of eighteen amino acids separated by less well-conserved fourteen amino acid sequences. Alternative splicing of exon 10, encoding the second C-terminal repeat, generates tau isoforms carrying three (3R) or four (4R) C-terminal repeats (Andreadis, 2005; Andreadis et al, 1992; Buee et al, 2000; Goedert & Jakes, 1990; Goedert et al, 1988).

The N-terminal region of tau functions as projection domain as it forms a link between the microtubule surface and the neural plasma membrane, other cytoskeletal elements and cytoplasmic organelles (Buee et al, 2000). Tau also binds to microtubules through its C-terminal repeats promoting their polymerisation and stabilisation. Splicing in of exon 10 creates tau isoforms with an extra microtubule-binding domain (4R tau) that are able to bind to microtubules more effectively than 3R tau, thereby stabilising them more effectively (Goedert & Jakes, 1990; Goode et al, 1997; Gustke et al, 1994). This is an important feature of tau because the differential stabilising abilities of 3R and 4R tau

isoforms are likely to contribute significantly to the level of plasticity in the brain (Ballatore et al, 2007; Buee et al, 2000).

Tau alternative splicing is regulated during development such that only the shortest tau isoform (0N3R) is expressed in embryonic stages and this continues to be expressed during adulthood. During early development, the high requirement for plasticity enables the formation of the various brain regions and their connections. At this stage, exclusive expression of the weaker microtubule-binding 3R tau is an advantage (Buee et al, 2000; Takuma et al, 2003). In contrast, expression of the other five isoforms of tau (1N3R, 2N3R, 0N4R, 1N4R and 2N4R) in the central nervous system (CNS) is specific to adult brain. During adulthood, the majority of tau isoforms contain a single N-terminal insert (1N), while the ratio of 3R:4R isoforms is approximately one. It is unclear how the different microtubule-binding affinities of the various tau isoforms affect their physiological roles (Buee et al, 2000; Hanger et al, 2009).

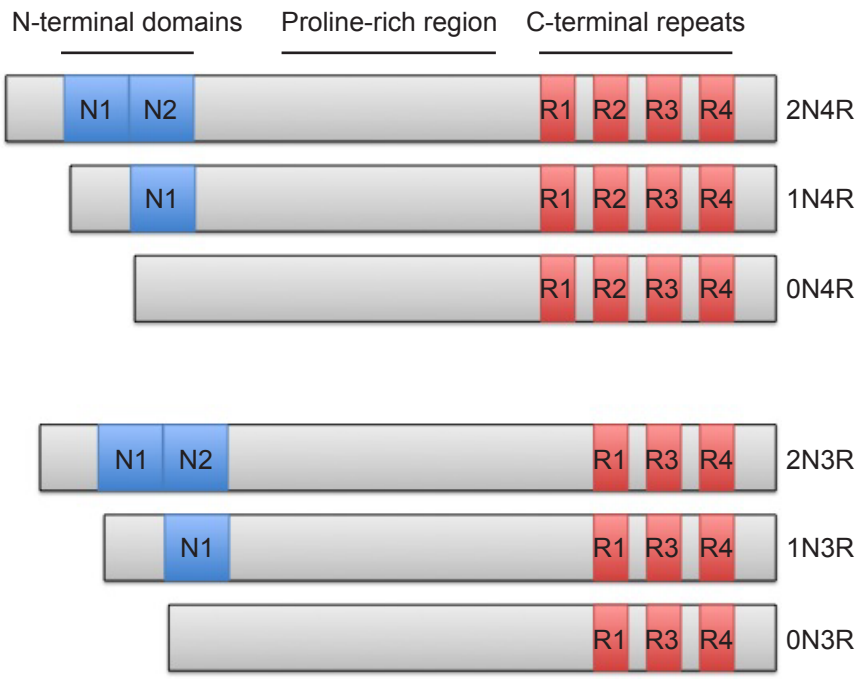

Figure 1.1 Human tau isoforms. Alternative splicing generates six tau isoforms ranging from 352 and 441 amino acids in length in adult human brain. These variants differ from each other by the number of N-terminal domains (2N, 1N or 0N, shown in blue) or C-terminal repeats (3R or 4R, shown in red) present in the protein. The N-terminal part of tau includes highly acidic twenty-nine amino-acid inserts (N) and a proline rich region. The C-terminal part of tau is characterised by conserved eighteen amino acids repeats (R) separated by fourteen amino acids fragments.

1.4 Tau function

In humans, MAPs are found in the majority of cell types, although tau is predominantly expressed in neurons (Buee et al, 2000). In glial cells, tau proteins are found mainly during pathological conditions, however, it is possible to detect tau mRNA and proteins in several peripheral tissues such as heart, kidney, lung, muscle, pancreas, testis, as well as in fibroblasts (Chin & Goldman, 1996; Ingelson et al, 1996; Vanier et al, 1998). Within neurons, tau is mainly localised to the axon where its primary function is to bind to and stabilise microtubules and to interact with other elements in the cell (Kanai et al, 1992).

The N-terminal inserts in tau are crucial for stabilisation and organisation of axons. These regions of tau extend from the microtubule surface to interact with other cytoskeletal elements forming the projection domain of the tau protein (Hirokawa et al, 1988). Thus, alternative splicing of exons 2 and 3 produces projection domains characterised by different lengths. It has been suggested that the size of the projection domain determines spacing of axonal microtubules and hence axonal diameter (Chen et al, 1992). Peripheral neurons, that typically project long axons with a large diameter, carry an additional N-terminal tau sequence encoded by exon 4A (Andreadis et al, 1992). The projection domain of tau protein also binds to spectrin and actin filaments (Carlier et al, 1984; Correas et al, 1990). This interaction allows microtubules to interconnect with other cytoskeletal components, such as neurofilaments, and may restrict the flexibility of the microtubules (Leterrier et al, 1982; Matus, 1994). Moreover, there is evidence that tau proteins interact with cytoplasmic organelles, such as mitochondria, endoplasmic reticulum, Golgi and the neural plasma membrane (Pooler et al, 2012).

Finally, tau may also play a role in the signal transduction pathway, in particular src-tyrosine kinase signalling. Tau protein can be phosphorylated by members of the src-family of tyrosine kinases, such as fyn, syk, c-Abl, lck and arg (Lebouvier et al, 2009; Scales et al, 2011). Phosphorylation of tau on T231 has been shown to reduce tau binding to the SH3 domain of fyn (Lee et al, 1998; Lee et al, 2004; Reynolds et al, 2008).

Through its C-terminal repeats, tau is able to bind to microtubules, to promote tubulin polymerisation, neurite outgrowth and axonal transport. The inter-region between the first and the second microtubule-binding region (R1-R2) is characterised by a high affinity for microtubule-binding and therefore it induces a high level of tubulin polymerisation. This R1-R2 inter-region is unique to 4R tau and confers a significantly increased microtubule-binding affinity to 4R tau compared to 3R tau (Goedert & Jakes, 1990; Goode et al, 1997; Gustke et al, 1994). Moreover, tau is important for the generation and maintenance of neurites *in vitro* (Yoshizaki et al, 2004). The binding of tau to microtubules within neurites is a critical step in the formation of the axon since it may promote additional binding and lead to neurite elongation (Ferreira et al, 1989). Neurite growth is directly modulated by changes in tau expression in cerebellar rat neurons *in vitro* (Caceres et al, 1991). In addition, tau plays a role in inhibition of kinesin-dependent fast axonal transport. The interference of tau with the ability of the kinesin motors to attach to microtubules has been demonstrated to be mediated by phosphorylation (Cuchillo-Ibanez et al, 2008; Mandelkow et al, 2003; Stamer et al, 2002). The microtubule-binding region may also be involved in functions other than microtubule assembly and growth. It might play an important role in development. In order to carry out this role, tau is specifically transported to the nucleus where it can bind RNA through its microtubule-binding domain (Brady et al, 1995; Kampers et al, 1996; Thurston et al, 1996).

1.5 Regulation of tau function by phosphorylation

In order to carry out its physiological function, the tau protein undergoes several post-translational modifications, in particular phosphorylation is known to regulate tau binding to microtubules (Lindwall & Cole, 1984). Phosphorylated tau reduces microtubule polymerisation compared to dephosphorylated tau. Inside neurons, tau exists in different phosphorylation states resulting from the activity of specific kinases and phosphatases. Although there are 85 sites that can be potentially phosphorylated on tau (45 serines, 35 threonines and 5 tyrosines), only 17 of these have been demonstrated so far to be phosphorylated on tau extracted from brain tissue from deceased healthy humans (Hanger et al, 2009).

Tau kinases phosphorylating serines and threonines can be broadly divided into two subgroups: the proline-directed kinases and the non-proline-directed kinases. Most of the kinases involved in tau phosphorylation are part of the proline-directed protein kinases targeting serine/proline and threonine/proline motifs on tau. These kinases phosphorylate tau at a large number of common residues and include mitogen activated protein kinase (MAPK), glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinases such as cdk5 (Baumann et al, 1993; Drewes et al, 1992; Hanger et al, 1992). The non-proline directed kinases phosphorylate tau within KXGS motifs present in the microtubule-binding domain. These kinases include microtubule affinity regulating kinase (MARK), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), cyclic-AMP-dependent kinase (PKA) and casein kinase 1 (CK1) (Baudier & Cole, 1987; Drewes et al, 1997; Jicha et al, 1999; Singh et al, 1995). In addition fyn, syk, c-Abl, lck and arg are thought to be the kinase responsible for tyrosine phosphorylation of tau (Lebouvier et al, 2009; Scales et al, 2011).

The activity of kinases is counterbalanced by phosphatases in the regulation of the phosphorylation status of tau. Protein phosphatases (PP) 1, 2A and 2B have been shown to dephosphorylate tau on different S/T residues *in vitro* (Liu et al, 2005; Yamamoto et al, 1988). In particular, PP2A activity on tau is much higher than the other phosphatases and this phosphatase is considered the main tau phosphatase (Goedert et al, 1995a).

1.6 Tauopathies

The term tauopathies refers to a collection of neurodegenerative disorders in which tau pathology is considered the major factor contributing to degeneration. In tauopathies, hyperphosphorylation of the tau protein leads to the formation of intraneuronal inclusions (Lee et al, 2001). The most common tauopathy is AD, while less frequent conditions caused by tau pathology are some of the FTLDs-tau (Table 1.2) (Dickson et al, 2011).

1.6.1 Alzheimer's disease

AD is the most common cause of senile dementia in humans. Patients affected by this pathology present symptoms characterised by progressive cognitive deterioration beginning with mild memory loss, behavioral changes and a decrease in everyday activities. With the progression of the condition, the clinical symptoms affect multiple cognitive and behavioral aspects such as language, learning, reasoning, attention, problem solving, emotion and memory (Walsh and Selkoe, 2004).

Amyloid plaques and NFTs are now widely accepted as the main pathological features that contribute to the neurodegenerative process in AD. Amyloid plaques are extracellular accumulations of the Aß peptide produced by cleavage of the amyloid precursor protein (APP), while NFTs are composed of intracellular depositions of hyperphosphorylated tau protein (Braak & Braak, 1996; St George-Hyslop, 2000). The APP gene is located on chromosome 21 and encodes for a transmembrane protein comprising a large extracellular and a short intracellular domain (Thinakaran & Koo, 2008). In healthy neurons the extracellular domain of APP undergoes a series of proteolytic cleavages by α-secretases, β-secretases and by the γ-secretase/presenilin complex (Wilson et al, 2003). During AD, the APP transmembrane protein undergoes an abnormal cleavage resulting in the release of Aβ1-40 and Aβ1-42. Aβ1-40 is the most common Aβ species, while Aβ1-42 is less abundant but the primary species found in Aβ plaques in AD brain (Evin et al, 2003). The formulation of the amyloid cascade hypothesis suggests that the aberrant processing of APP leading to the production of Aβ is the initial cause of the pathological processes in AD and that this precedes the formation of tau tangles. Abnormal processing of APP and subsequent elevated Aβ levels are thought to trigger NFT formation which then leads to terminal degeneration of the affected neurons, giving rise to the clinical symptoms (Hardy & Higgins, 1992; Selkoe, 1991). Mutations in the APP gene or in the presenilin genes affect the metabolism and production of Aβ1-42 and cause rare early-onset familial AD accounting for only 10% of cases (Hashimoto et al, 2000). Intracellular soluble Aβ species may also be important in the pathogenesis of AD. The release of toxic Aβ species can occur in the intracellular space since APP is found on the membrane of organelles such as the Golgi network, endoplasmic reticulum and lysosomal, endosomal

and mitochondrial membranes (Mizuguchi et al, 1992; Xu et al, 1995).

The second neuropathological feature of AD is the deposition of intracellular NFTs made of hyperphosphorylated tau protein. These aggregated filaments are composed of an equimolar ratio of 3R and 4R tau isoforms (Goedert et al, 1992). These intracellular depositions can be distinguished as either paired helical filaments (PHFs) or straight filaments. PHFs are composed of two strands of filaments twisted around one another similar to a helix, while straight filaments lack the helical shape. Both of these intracellular inclusions are composed predominantly of insoluble, abnormally highly phosphorylated tau and are thought to be responsible for neuronal death (Crowther, 1991; Iqbal et al, 1984; Mandelkow et al, 2007). Tau hyperphosphorylation occurs on many residues that are not phosphorylated in healthy neurons and also on usually phosphorylated residues at a much higher stoichiometry (Hanger et al, 2009). The abnormal phosphorylation of tau leads to neurodegeneration by deposition of NFTs, impairment of axonal transport and synaptic loss (Morfini et al, 2009; Terry et al, 1991).

1.6.2 Frontotemporal lobar degenerations

The frontotemporal lobar degenerations (FTLDs) are a group of dementia disorders sharing common clinical symptoms and molecular features of pathogenesis. In contrast with AD, which presents predominantly as memory loss, the clinical symptoms of FTLDs are associated with changes in personal and social conduct, severe behavioural and language disturbances, and motor symptoms with cognitive dysfunction appearing later (Neary et al, 1998). The neurodegeneration process in FTLDs affects the frontal and temporal lobes of the brain leading to cortex atrophy, neuronal loss and gliosis. The molecular pathogenesis is caused by an abnormal protein deposition that differs for protein type, inclusion morphology and distribution depending on the disease (Josephs, 2008). Three proteins have been identified to be independently responsible for the mechanism of neurodegeneration in FTLDs: tau, the transactive response DNA binding protein of 43 kDa (TDP-43) and the tumour-associated protein fused in sarcoma (FUS) (Hutton et al, 1998; Kwiatkowski et al, 2009; Neumann et al, 2006).

The FTLDs associated with tau (FTLDs-tau) consist of a group of diseases in which the major pathogenic mechanism involves the microtubule-associated protein tau. The FTLDs-tau pathologies include frontotemporal degeneration with parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease (PiD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grains disease (AGD), sporadic multisystem tauopathy with globular inclusions (MST) and diffuse neurofibrillary tangle dementia with calcifications (DNTC) (Bigio et al, 2001; Braak & Braak, 1989; Dickson, 1999; Dickson, 2001; Kosaka, 1994; Wszolek et al, 2006).

1.6.2.1 Frontotemporal dementia with parkinsonism linked to chromosome 17

Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP- 17) is a form of dementia caused by autosomal-dominant mutations in the tau gene leading to the formation of abundant tau inclusions in neurons and/or glia cells. These exonic and intronic mutations in the tau gene were first identified by Hutton et al (1998) in FTDP-17 patients. The pathogenic mutations identified in the tau gene can be grouped according to their positions in the gene, which also defines the resultant pathology. Moreover, depending on their nature, tau mutations can have multiple effects including alteration of microtubule assembly, promotion of tau filament formation and alteration of tau mRNA splicing and isoform expression.

Tau gene mutations include missense, deletion and silent mutations and the majority of these are found in the coding region of tau. Most coding region mutations are located in the microtubule-binding region of tau and several of these lead to a reduction in the affinity of tau for microtubules, therefore decreasing the ability of mutant tau to promote microtubule assembly (Dayanandan et al, 1999; Hong et al, 1998). For example, the missense mutations P301L and P301S, are included in exon 10 encoding the second microtubule-binding region of tau. In particular, the P301L mutation causes the selective incorporation of 4R into tau inclusions only in brain regions affected by FTDP-17 (Hong et al, 1998). Other mutations are thought to introduce or remove potential phosphorylation sites that could lead to functional changes in the microtubule-binding properties of tau (Hasegawa et al, 1998). In addition, a large number of

missense mutations are located outside exon 10, examples include G272V, V337M, G389R and R406W (Hutton et al, 1998).

In contrast, intronic mutations in the tau gene are located close to the splice-donor site of the intron downstream of exon 10. These mutations affect exon 10 splicing, leading to an altered expression of 3R and 4R tau isoforms (D'Souza et al, 1999; Giaccone et al, 2005; Hong et al, 1998; van Swieten et al, 2007). Importantly, several mutations result in increased expression of 4R tau and the formation of tau deposits consisting mainly of 4R tau, such as N279K (Grover et al, 2003). The most prevalent familial tau mutation leading to FTDP-17 appears to be P301L (Bird et al, 1999; Kodama et al, 2000; Mirra et al, 1999; Rizzu et al, 1999; Tanaka et al, 2000; Zekanowski et al, 2003). Additional mutations have often occurred as independent events in several families, including R406W. The R406W mutations in tau leads to a slow rate of disease progression lasting up to 25 years, compared to an expected duration of illness of 10 years (Rademakers et al, 2003) seen for other mutant forms.

1.6.2.2 Pick's disease

Pick's disease (PiD) is a rare cause of frontal lobe dementia usually associated with a presenile dementia with age of onset younger than 65 years (Kertesz, 2010). The neuropathological feature characteristic of PiD is the presence of intra-neuronal inclusions enriched in hyperphosphorylated 3R tau, also known as Pick bodies (Buée & Delacourte, 1999; de Silva et al, 2006). These tau depositions are characterised by a rounded appearance and are localised in the cytoplasm of neurons (Dickson, 2001). Differences in the molecular composition of the tau deposits are linked to differences in their argyrophilic properties in reducing silver. The Pick bodies are strongly argyrophilic using the Campbell-Switzer method that is related to deposits containing 3R tau, while those staining with Gallyas are linked to those containing 4R tau (Uchihara et al, 2005). Finally familial cases of PiD have been confirmed to be caused by mutations in the tau gene (Bronner et al, 2005; Murrell et al, 1999). In particular, the G272V mutation and other tau mutations in exons 9 and 11-13 cause hereditary Pick's disease and affect the biochemical composition of Pick bodies.

Levels of soluble tau are higher in PiD compared to AD, however, its phosphorylation levels are lower compared to AD (van Eersel et al, 2009). The two disorders also display a different phosphorylation profile on specific residues. In PiD, soluble tau is not highly phosphorylated on S396, S404 and S422 compared to AD, whereas the pT181 epitope displays more prominent phosphorylation in PiD compared to AD (van Eersel et al, 2009). In addition, PiD is characterised by dephosphorylation of S262 (Bronner et al, 2005).

1.6.2.3 Progressive Supranuclear Palsy and Corticobasal Degeneration

Progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) are associated with a variety of clinical symptoms arising from the different distribution of the pathology rather than different histopathologic appearance of the pathology.
Both PSP and CBD present with neuronal, oligodendroglial, and astrocytic lesions characterised by the deposition of hyperphosphorylated 4R tau aggregates (Chambers et al, 1999). The differences in these two disorders are in the morphology and proportion of the toxic lesions (Dickson, 1999; Komori et al, 1998). The tau inclusions characterising PSP are large, globular deposits and the astrocytic lesions are represented by tuft-shaped aggregates (Yamada et al, 1992). In contrast, CBD pathology presents with fewer oligodendroglial inclusions and a specific type of astrocytic lesions, known as astrocytic plaques (Feany & Dickson, 1995).

PSP and CBD show a differential production of low molecular weight tau fragments found in 4R tau isoforms only, suggesting the occurrence of different proteolytic events (Arai et al, 2004). A 35 kDa tau fragment lacking of the N-terminus region of the protein is predominant in PSP brains, while in CBD brains the 37 kDa tau species are more common (Arai et al, 2004; Wray et al, 2008). The number of phosphorylated residues on tau identified in PSP brains is substantially lower than that in AD brains (Table 1.3) (Wray et al, 2008). Genome-wide association analyses of PSP and CBD patients have demonstrated that these pathologies might have common genetic risk factors in the H1 haplotype, a region of complete linkage disequilibrium encompassing the entire coding sequence of tau and several other genes (Conrad et al, 1997; Di Maria

et al, 2000; Pittman et al, 2006; Pittman et al, 2004). Very recently novel genetic risk factors for PSP have been associated with the STX6 gene involved in the vesicle fusion at the Golgi, the EIF2AK3 gene for the endoplasmic reticulum unfolded protein response and the MOBP gene for a myelin structural component (Höglinger et al, 2011).

	Brain regions	Clinical symptoms	Tau aggregates	Cell types	Tau isoforms	3R:4R ratio
AD	Cortex and limbic lobe	Memory loss, confusion, behavioural changes, language impairment	NFTs (helical or straight filaments)	Neurons	3R, 4R	1:1
FDTP-17	Fronto-temporal cortex, basal ganglia, brainstem	Psychosis, severe behaviour and language disturbances, cognitive dysfunction, motor symptoms	AD-like filamentous inclusions	Neurons, glia	3R, 4R	1:2 1:1 2:1
PiD	Fronto-temporal cortex, limbic lobe	Psychosis, severe behaviour and language disturbances, cognitive dysfunction	Argyrophilic rounded inclusions	Neurons, glia	3R	3:1
PSP	Basal ganglia, brainstem, cerebellum	Atypical motor dysfunction, vision impairment, mild cognitive dysfunction	Large and globular aggregates, astrocytic tuft-shaped aggregates	Neurons, oligodendroglia, astrocytes	4R	1:2-4
CBD	Fronto-temporal cortex, basal ganglia	Atypical motor dysfunction, language disturbances, mild cognitive dysfunction	Large and globular aggregates, astrocytic plaques	Neurons, oligodendroglia, astrocytes	4R	1:2

Table 1.2 Main tauopathies. The major types of tauopathies are listed together with the brain regions affected, their clinical symptoms, the types of tau aggregates present, the cell types affected, the tau isoforms composing the aggregates and the proportion of tau isoforms present in the brain.

1.7 Pathogenic tau post-translational modifications

The physiological function of tau is predominantly regulated by post-translational modifications. Disruption to these modifications on tau might play a central role in tau dysfunction and aggregation in the tauopathies. Therefore, understanding the post-translational regulation of tau may be essential to identify the origin of tau aggregate formation (Buee et al, 2000).

1.7.1 Tau hyperphosphorylation

The normal physiological activity of tau to bind microtubules within neurons is regulated by specific phosphorylation events (Lindwall & Cole, 1984). However, there is growing evidence that during tauopathies the normal regulatory role of phosphorylation may be disrupted leading to disease. It is thought that a series of aberrant phosphorylation events contribute to the formation of tau aggregates which sequester tau away from its physiological function and allow precipitation in neurons leading to toxicity (Alonso et al, 2001; Ballatore et al, 2007; Lauckner et al, 2003). Whether hyperphosphorylation of tau is due to an increase of tau kinase activity or to a decrease in the activity of tau phosphatases is still debated. It is unclear if the pathological tau kinases correspond to those regulating the physiological function of tau or if new kinases participate in the aberrant tau phosphorylation in disease. In addition, previous work demonstrates that phosphatase activity, in particular PP2A, is decreased in AD brains (Gong et al, 1993; Liu et al, 2005). Another possibility is that tau might become a better substrate for phosphorylation through an altered conformation or mislocalisation. In healthy neurons tau is localised to the axon, however in brains affected by tau pathology tau is mislocalised to the somatodendritic compartments (Hoover et al, 2010).

The pathogenic hyperphosphorylation events on tau result in the attachment of 3 to 5 times more phosphate compared to tau in physiological condition (Ksiezak-Reding et al, 1992). This difference plays a critical role during pathogenesis because it alters the microtubule-binding affinity of tau. Indeed, the tau protein becomes

hyperphosphorylated often within its microtubule-binding domains, reducing the ability to bind to microtubules (Amniai et al, 2009; Ballatore et al, 2007; Iqbal et al, 2009). During pathogenesis, the aberrant phosphorylation events lead to the formation of tau depositions that precipitate in neurons. There are a number of ways in which these neuronal deposits may cause cell death. The intracellular deposition of tau filaments may form a physical impediment that compromises cellular functions including distribution and number of organelles as well as proteasome activity (Ballatore et al, 2007; Iqbal et al, 2009). In addition, the sequestration of tau into aggregates causes the destruction of the microtubule network and consequently affects axonal transport and synaptic integrity (Morfini et al, 2009; Terry et al, 1991). Phosphorylation of tau is known to decrease the affinity of tau for microtubule-binding resulting in the detachment of tau from microtubules that no longer polymerise (Amniai et al, 2009). Microtubules together with kinesins are essential in the anterograde axonal transport that enables essential components to travel from their sites of synthesis in the cell body to synaptic locations along the axon (Dixit et al, 2008). As shown by cell culture studies, phosphorylated tau leads to the inhibition of anterograde axonal transport due to interference with kinesins (Stamer et al, 2002). Therefore, the relationship between axonal transport and tau is closely associated with the phosphorylation status of tau. Moreover, expression of tau in cultured neurons causes drastic changes in dendritic spine morphology leading to synapse loss and cell death (Hall et al, 2000). *In vivo* experiments have shown that human tau aggregation results in an age-dependent perturbation in basal synaptic transmission in areas of the brain associated with neurodegeneration (Polydoro et al, 2009).

1.7.2 Other post-translational modifications of tau

Beyond phosphorylation, the tau protein also undergoes other post-translational modifications, some of which have been postulated to have roles in the formation of pathogenic tau tangles (Buee et al, 2000; Gong et al, 2005).

Firstly, tau glycosylation is implicated in PHF formation and stabilisation during disease. *N*-glycosylation of tau occurs only during disease and leads to the stabilisation

of the PHF conformation of tau (Wang et al, 1996). In contrast, *O*-glycosylation is thought to modulate tau function in healthy neurons by the addition of an *O*-linked N-acetylglucosamine residue on serines or threonines in the proximity of proline residues (Haltiwanger et al, 1992). *O*-glycosylation of tau has been suggested to mediate the interactions between tau and tubulin, leading to an opposite effect to tau phosphorylation (Liu et al, 2004). Finally *O*-glycosylation has been reported to be upregulated in pathogenesis (Griffith & Schmitz, 1995).
Moreover, when assembled into PHFs, tau has been shown to be ubiquitinated. The attachment of ubiquitin is a signal for degradation of short-lived, abnormal or damaged proteins. Tau ubiquitination probably suggests an attempt by the cell to eliminate the toxic PHFs species (Bancher et al, 1991).

It has also been suggested that PHF insolubility may be related to glycation of tau, the reaction between the N-terminal group of an amino acid side chain and the carboxyl end of reducing sugars. Tau glycation occurs specifically during pathogenesis and leads to a cross-linking reaction causing the formation of insoluble aggregates of proteins (Ko et al, 1999; Liu et al, 2002). On PHF-tau thirteen residues of lysine are found to be glycated in one of the microtubule-binding domains, therefore their modification could result in a decreased interaction of tau with tubulin (Ledesma et al, 1995).

Finally, tau has been shown to undergo cleavage by a number of proteases such as caspases, thrombin and calpain. The resulting fragments appear to have a higher propensity for aggregation when compared to full-length tau (Hanger & Wray, 2010; Novák, 1994). Whereas, PHF-tau has been demonstrated to be extremely resistant to degradation by the calcium-dependent protease calpain compared to normal tau protein. Only a partial proteolysis was obtained with a higher concentration of calpain forming mainly N-terminal tau fragments. The resistance of abnormal filaments to proteolysis is thought to be associated with the conformation of tau proteins into PHF, rather than with hyperphosphorylation (Litersky & Johnson, 1992). In particular, calpain-digestion sites might be buried in the core of filaments and could become inaccessible to the protease (Yang & Ksiezak-Reding, 1995). Beyond exhibiting strong aggregation properties, tau fragments are capable of seeding the aggregation of both cleaved and full-length tau (Kovacech & Novak, 2010).

1.8 Tau kinases as major contributors to pathogenesis

Hyperphosphorylation of tau is thought to be one of the major contributors leading to tau aggregation and toxicity during tauopathies (Alonso et al, 2001; Ballatore et al, 2007; Lauckner et al, 2003). Potential therapeutic strategies against tauopathies have focused on reducing tau phosphorylation through inhibition of specific protein kinases (Lau et al, 2002). This approach aims to reduce tau aggregation and associated neuronal loss slowing neurodegeneration (Churcher, 2006). Recently, the validity of this approach has been demonstrated by successful experiments in transgenic animals. In fact, it has been demonstrated that tau kinase inhibitors can reduce tau hyperphosphorylation and prevent motor deficits in transgenic mice over-expressing human tau (Lewis et al, 2000). Calpain inhibitor prevents cdk5 activation and decreases tau phosphorylation improving neuronal function in a rat model of tau hyperphosphorylation (Hung et al, 2005). Furthermore, inhibition of GSK3β by lithium ions correlates with reduced tauopathy and neurodegeneration *in vivo* (Munoz-Montano et al, 1997; Nakashima et al, 2005; Noble et al, 2005). Therefore, it is crucial to identify the protein kinases and phosphatases that regulate tau phosphorylation in order to find further targets and optimise current therapeutic approaches.

There is no *in vivo* evidence of which kinases are involved in the production of pathogenic tau hyperphosphorylation in AD brains. However, many kinases have been shown to phosphorylate tau *in vitro* (Anderton et al, 2001; Cruz & Tsai, 2004; Feijoo et al, 2005; Hanger et al, 2009; Hanger et al, 2007; Mandelkow et al, 2004). The potential major protein kinases that mediate pathological phosphorylation of tau include GSK3β, cdk5, MAPK, PKA and CK1δ (Hanger et al, 2009). *In vitro* phosphorylation studies with purified protein kinases and recombinant tau have shown that GSK3β can phosphorylate at least 37 of the identified tau phosphorylation sites, PKA up to 27 sites and CK1δ at least 39. A combination of GSK3β, PKA and CK1δ can phosphorylate 38 sites known to be phosphorylated in PHF-tau extracted from AD brains (Hanger et al, 2009; Hanger et al, 2007).

1.8.1 Glycogen synthase kinase 3β

GSK3β is an evolutionary conserved S/T protein kinase initially described for its role to phosphorylate and inhibit glycogen synthase (Rylatt et al, 1980). It is an important component of signalling systems and targets a large number of substrates, including metabolic proteins, growth factors, cytoskeletal proteins, transduction and transcription factors and other signaling molecules (Medina et al, 2011). Expressed in all tissues, GSK3β is abundant in the brain, in particular in neurons, where it plays a fundamental role in neuronal signaling pathways (Leroy & Brion, 1999). Different aspects of neuronal and glial development are regulated by GSK3β, such as morphogenesis, axonal polarity, synaptogenesis and cell survival (Grimes & Jope, 2001). Among the processes controlled by GSK3β, regulation of cytoskeletal dynamics is one of the most critical since it is involved in neuronal plasticity (Cohen & Frame, 2001; Frame & Cohen, 2001).

GSK3β phosphorylates several proteins that are critical components of the cytoskeleton and tau was found to be one of them (Hanger et al, 1992). A link between GSK3β and the major neuropathological mechanisms associated with AD and tauopathies has been suggested (Hanger & Noble, 2011). In particular, GSK3β may contribute to the abnormal phosphorylation of tau observed in NFTs (Hanger et al, 1992). Furthermore, GSK3β has been shown to phosphorylate tau both *in vitro* and in cultured cells at epitopes known to be phosphorylated in post-mortem AD brain tissue (Hanger et al, 2007; Hanger et al, 1992; Lovestone et al, 1994b). In particular, over-expression of GSK3β in cells causes a dramatic change in tau phosphorylation at a number of residues such as pS202/T205 (Lovestone et al, 1996; Wagner et al, 1996). Transgenic mice over-expressing an inducible form of GSK3β show a higher level of tau phosphorylation and neurodegeneration than control brains (Lucas et al, 2001). In contrast, tau phosphorylation and tangle load are reduced in neurons exhibiting neurofibrillary pathology after lithium-induced inhibition of GSK3β *in vitro* and *in vivo* (Lovestone et al, 1999; Munoz-Montano et al, 1997; Noble et al, 2005). Finally, GSK3β has been demonstrated to be present at increased levels in AD brains where it has been found to co-localise with NFTs (Pei et al, 1999; Yamaguchi et al, 1996).

All together this evidence suggests that GSK3β is a significant contributor to tau toxicity and is an excellent target for the development of inhibitors of tau hyperphosphorylation. Indeed, several pharmaceutical companies now have inhibitors of GSK3β under development as therapeutic agents for tauopathies. However, it is not clear whether inhibition of GSK3β alone would be sufficient to alleviate disease and it is necessary to identify further kinases that contribute to tau pathology since they may be considered as additional therapeutic candidates (Hanger et al, 2009).

1.8.2 Cyclic-AMP-dependent kinase

The cAMP-dependent protein kinase PKA is part of a family of enzymes whose activity is dependent on cellular levels of cyclic AMP (cAMP). PKA is a holoenzyme composed of two regulatory and two catalytic subunits. An increased level of cAMP leads to the activation of the kinase. Binding of cAMP to the regulatory subunits alters their affinity for the catalytic subunits that are released as two active monomers (Taylor et al, 1990). PKA is expressed in kidney, liver, muscular system and CNS where it is mainly involved in the regulation of glycogen, sugar, and lipid metabolism.

Several studies have shown that the tau protein is also a substrate for PKA (Litersky & Johnson, 1992; Pierre & Nunez, 1983). *In vitro* phosphorylation of recombinant human tau by PKA results in decreased electrophoretic mobility of tau on SDS-PAGE and in a 92% decrease in the rate of tau-induced microtubule assembly (Robertson et al, 1993; Scott et al, 1993; Singh et al, 1994). Tau phosphorylation by PKA occurs in three sequential stages characterised by increased phosphorylation and decreased electrophoretic mobility of tau (Scott et al, 1993). Up to 27 phosphorylation sites were identified as PKA targets on recombinant tau, two of which were located within microtubule-binding domains: S214, S324, S356, S409, and S416 (Scott et al, 1993). Moreover, it has been shown that PKA phosphorylation of tau on S356 and S409 induces the PHF kinase associated with tau filaments to sequentially phosphorylate recombinant tau *in vitro* (Jicha et al, 1999; Vincent & Davies, 1992).

Furthermore, PKA has been demonstrated to be involved in the regulation of tau

degradation. In particular, tau proteolysis by calpain is significantly reduced by PKA phosphorylation, possibly on specific sites (Litersky & Johnson, 1992). During tauopathies, abnormal phosphorylation could result in a protease-resistant tau population that may contribute to the formation of PHFs.

1.8.3 Casein kinase 1δ

Casein kinase 1 (CK1) encompasses a large family of evolutionary conserved S/T protein kinases found in a variety of subcellular locations (Vielhaber & Virshup, 2001). *In vitro* studies have revealed that CK1 proteins phosphorylate substrates with a negatively charged region N-terminal to the site of phosphorylation, in particular substrates containing the recognition motifs S/T(P)-X-X-S/T (Flotow & Roach, 1991). The CK1 family is involved in regulating a large number of cellular processes, including cell cycle progression in oocytes, RNA metabolism and synaptic vesicle formation. In particular, CK1δ may regulate the cell response to DNA damage (Vielhaber & Virshup, 2001).

The CK1 family has been shown to phosphorylate the tau protein and it is thought to be one of the principal families of phosphotransferases associated with tau filaments in tauopathies (Hanger et al, 2007; Singh et al, 1995). Evidence in cultured cells shows that over-expression of CK1δ significantly increases tau phosphorylation of S202, T205, S396 and S404 and reduces the fraction of tau bound to microtubules. These results suggest that CK1δ can phosphorylate tau at sites that modulate its binding to microtubules (Li et al, 2004). Moreover, members of the CK1 family correlate spatially with neurofibrillary lesions in tauopathies (Kuret et al, 1997). In particular, one isoform, CK1δ, shows particularly robust co-localisation with granulovacuolar degeneration bodies in the hippocampus (Ghoshal et al, 1999; Schwab et al, 2000). Furthermore, CK1 levels are elevated in the hippocampus of AD brains, with CK1δ being elevated over thirty fold (Ghoshal et al, 1999; Yasojima et al, 2000). The localisation and expression pattern of the CK1 protein kinase family leads to the hypothesis that CK1δ has a key role in the hyperphosphorylation of tau and in the formation of tau pathology *in vivo*.

1.8.4 Dual-specificity tyrosine-regulated protein kinase 1A

DYRK1A belongs to a family of dual-specificity tyrosine-regulated protein kinases (DYRKs) that possess dual substrate specificities. They are characterised by S/T phosphorylation activity as well as auto-phosphorylation activity on tyrosine residues (Y321 residue for self-activation) with a preferred phosphorylation consensus motif of RPX(S/T)P (Kentrup et al, 1996; Marti et al, 2003). In humans DYRK1A is highly expressed at embryonic stages and also during adulthood in the CNS, suggesting an involvement in the physiology of the CNS. It has been suggested that DYRK1A may control proliferation and maturation events during development and motor function in the adult (Marti et al, 2003). DYRK1A knockout mice show a general delay in foetal development and are embryonic lethal suggesting vital and non-redundant biological function of DYRK1A (Fotaki et al, 2002). DYRK1A has also been identified as regulator of microtubule stability in growing axons via a priming action on GSK3β phosphorylation of MAP1B (Scales et al, 2009).

Importantly, it has been shown that Down syndrome (DS) patients constantly suffer early-onset AD and that the DYRK1A gene is localised on the human chromosome 21 within the DS critical region (Altafaj et al, 2001; Ryoo et al, 2007). This chromosomal region is relatively small but when present in three copies it is sufficient to produce DS, the major cause of mental retardation in humans. Therefore, the position of the DYRK1A locus makes this kinase a good candidate gene to contribute to the aberrant brain development underlying mental retardation in DS and a factor that may cause AD. The CNS of DS patients shows a large number of alterations, including a reduction of brain size, abnormal neuronal migration, differentiation and density, affecting several brain regions (Altafaj et al, 2001). Both DS and AD have common pathological hallmarks such as amyloid plaques and NFTs. Higher levels of DYRK1A have been found in DS brains and its presence has been detected in NFTs from patients with sporadic AD (Liu et al, 2008; Wegiel et al, 2008).

Transgenic mice over-expressing DYRK1A show specific motor and cognitive defects similar to those seen in DS patients (Altafaj et al, 2001; Marti et al, 2003; Ryoo et al, 2007). Moreover, in neuronal cultures over-expression of DYRK1A has been shown to

contribute to synaptic dysfunction reducing synaptic vesicle endocytosis (Kim et al, 2010). DYRK1A has been shown to phosphorylate the tau protein *in vivo* mainly in its proline-rich region. The known phosphorylation sites on tau *in vitro* that may play a key role in the loss of microtubule assembly and in the formation of NFTs are S202, T205, T212 and S404 (Hanger et al, 2009; Liu et al, 2008; Ryoo et al, 2007). There is also evidence suggesting that over-expression of DYRK1A in DS brain induces tau hyperphosphorylation. In particular, DYRK1A phosphorylation of T212 residue promotes GSK3β-mediated phosphorylation of tau that ultimately leads to tau lesions in the brain (Woods et al, 2001). In conclusion, DYRK1A could be a novel potential therapeutic target for the treatment of AD in both DS and AD patients.

1.8.5 Multiple kinases

It is becoming clear that no single kinase or phosphatase is responsible for all of the abnormal tau phosphorylation observed in tauopathies. Recent *in vitro* studies have suggested that multiple kinases are likely to be involved in tau phosphorylation and that specific kinases may prime tau for phosphorylation by others inducing a kinase cascade. Indeed, CK1, DYRK1A and cdk5 have been suggested to prime tau for subsequent phosphorylation by GSK3β (Hanger et al, 2007; Li et al, 2006; Woods et al, 2001). Therefore, targeting the inhibition of a priming kinase may be another promising therapeutic strategy to reduce the overall level of tau phosphorylation.

1.9 Phosphorylation sites on tau contributing to disease

During disease, hyperphosphorylation of tau results in the loss of its physiological function and in the gain of a toxic function (Alonso et al, 2001; Ballatore et al, 2007; Lauckner et al, 2003). The mechanisms by which tau kinases and their phosphorylation sites lead to neuronal toxicity are still debated. Importantly, the phosphorylation sites that are crucial for the physiological and pathological role of tau *in vivo* remain unknown.

An increase of phosphorylation of residues in the microtubule-binding domains of tau and the flanking regions is reported to have a large effect on the ability of tau to bind to microtubules (Amniai et al, 2009). Hyperphosphorylation of sites usually involved in the regulation of the microtubule-binding activity causes the loss of physiological function of tau. Thus, defects in microtubule assembly and polymerisation lead to axonal transport impairment and synaptic loss (Morfini et al, 2009; Terry et al, 1991). On the other hand, phosphorylation at specific residues not normally phosphorylated in physiological conditions may cause conformational changes of tau (Jeganathan et al, 2008). Indeed, recent studies have identified 45 phosphorylated sites on tau extracted from AD brains in contrast with only 17 extracted from healthy brains (Table 1.3) (Hanger et al, 2009; Hanger et al, 2007; Lebouvier et al, 2009; Morishima-Kawashima et al, 1995). The conformational changes on tau might lead to inaccessibility for phosphatases, to increased affinity for kinases or to impaired tau physiological functions (Fischer et al, 2009).

In conclusion, the hyperphosphorylation process that occurs on tau during pathogenesis includes an increased phosphorylation of residues of tau that normally participate in the physiological role of tau, but also includes the phosphorylation of additional sites that are not involved in the microtubule-binding function. However, the role that specific phosphorylated residues play in disease remains elusive.

Residue number	Control brain	AD PHF-tau	PSP tau	MAPK	GSK3β	PKA	CK1δ
T17							★
Y18		A					
Y29							
T30							
T39							
S46	★	A	★	★	★		★
T50					★		★
T52							
S56							
S61							
T63							
S64							
S68		★					
T69		★		★	★		
T71		★					
T76							
T95							★
T101							★
T102							★
T111							
S113		★					★
T123		A					
S129							
S131							★
T135							
S137							
T149					★		★
T153		A			★		
T169							★
T175		★		★	★		
T181	★	★	★	★	★		
S184		★			★		★
S185		★		★			
S191		★					
S195					★	★	
Y197		★					
S198	★	★			★	★	★
S199	★	★			★	★	
S202	★	★	★	★	★	★	
T205	★	A	A	★	★	★	
S208		★					★
S210		★			★	★	★
T212	★	★	A	★	★	★	★
S214		★	A		★	★	★
T217	★	★	★	★	★	★	
T220					★	★	
T231	★	★	★	★	★	★	
S235	★	★	★	★	★	★	
S237		★			★		★
S238		★					★
S241					★		★
T245				★	★	★	
S258		★			★	★	★
S262		★	A		★	★	★
T263							★
S285					★		★
S289		★			★		★

Residue number	Control brain	AD PHF-tau	PSP tau	MAPK	GSK3β	PKA	CK1δ
S293						★	
S305				★	★	★	★
Y310							
S316							
T319							
S320				★		★	
S324					★	★	
S341							★
S352					★	★	★
S356		★		★	★	★	★
T361							★
T373					★		★
T377							
T386							★
Y394		★					
S396	★	★	★	★	★		★
S400	★	★	★		★		
T403		★	★				
S404	★	★	★	★	★		★
S409		★	A	★	★	★	
S412	★	★				★	★
S413	★	★	A		★	★	★
T414	★	★					★
S416	★	★				★	★
S422		★	A	★		★	
T427		★					
S433		★					★
S435		★				★	★

Table 1.3 Phosphorylated sites on human tau. The phosphorylation sites on human tau extracted from control brains, AD brains and PSP brains are listed together with those by major S/T tau kinases MAPK, GSK3β, PKA and CK1δ. The residue numbering refers to the 2N4R human tau isoform. The star indicates sites identified by direct means (mass spectrometry or Edman degradation) and "A" indicates sites identified using phospho-specific tau antibodies (http://cnr.iop.kcl.ac.uk/hangerlab/tautable).

1.10 *Drosophila melanogaster* to study neurodegeneration

Most work investigating the phosphorylation of tau has been performed *in vitro* with purified proteins; *in vivo* validation of these findings is required. Therefore, it is essential to understand the importance of the candidate kinases that produce tau toxicity and their activity on the predicted sites *in vivo*. *Drosophila melanogaster* has emerged as an excellent model system for studying human neurodegenerative disease (Moloney et al, 2010; Muqit & Feany, 2002). Beyond the short lifespan and the useful genetic tools available, *Drosophila* has a complex nervous system and clear orthologs exist for 70% of the disease-related loci found in humans (Reiter et al, 2001).

Meaningful fly models of tauopathy have been created by expressing human tau in the *Drosophila* photoreceptor neurons (Bonini & Fortini, 2003). Examination of the degeneration in the *Drosophila* eye has numerous advantages including dispensability for fly viability and ease of use in degeneration screens due to loss of crystalline-like eye structure. Therefore, the *Drosophila* eye represents a quick readout of neurodegeneration that can be applied in large-scale screenings. Moreover, expression of human tau in *Drosophila* photoreceptor neurons results in neurodegeneration that bears many of the hallmarks of tauopathies including age dependency, presence of abnormally phosphorylated tau and in some cases tau aggregates (Jackson et al, 2002; Nishimura et al, 2004).

The *Drosophila* models produced so far have focused on identifying *Drosophila* genes that can modify human tau toxicity (Chatterjee et al, 2009; Chau et al, 2006; Jackson et al, 2002; Shulman & Feany, 2003; Steinhilb et al, 2007a; Steinhilb et al, 2007b; Wittmann et al, 2001). To date there has been little work on understanding the causes of human tau-induced neurodegeneration in *Drosophila.* However, it appears that the sequential activation of the TOR signalling pathway enhances tau-induced neurodegeneration and drives cell cycle activation leading to apoptosis in fly cells expressing human tau (Khurana et al, 2006). A genetic screen for modifiers of tau-induced neurodegeneration revealed endogenous *Drosophila* genes, primarily kinases and phosphatases that enhance or suppress human tau toxicity (Shulman & Feany, 2003; Steinhilb et al, 2007b). Wittmann et al (2001) first created an *in vivo* model of

tauopathy that showed neurodegeneration without the formation of NFTs. Indeed, this study also demonstrated that expression of human 0N4R tau in the fly CNS led to progressive vacuolisation and degeneration of neurons in the cortex without the formation of tau aggregates. Moreover, the FTDP-17 associated tau mutations R406W and V337M, increased human tau toxicity in flies, showing more severe neurodegeneration in the fly brain (Wittmann et al, 2001). The R406W mutation also resulted in increased eye degeneration and phosphorylation of tau by endogenous kinases on residues T181, S202, T205, T231 and S235 (Chau et al, 2006). However, it is not clear how the R406W mutation influences tau-mediated toxicity in flies. It was also shown that human 0N4R tau expressed in the fly CNS or eye is phosphorylated by endogenous *Drosophila* kinases on epitopes directly associated with tauopathies, including T181, S199, S202, T205, T212, S214, T217, T231, S235, S396 and S404 (Steinhilb et al, 2007a; Wittmann et al, 2001). As the fly ages, tau phosphorylation increases and accumulates in areas of degeneration (Wittmann et al, 2001).

The ease of genetic manipulation on *Drosophila* allows investigation of the effects of tau mutations on phosphorylation and toxicity of human tau in flies. For example, individual point mutations in 0N4R tau that inhibit phosphorylation do not affect the neurodegeneration caused by wild type tau in the fly eye. However, when human tau carries multiple mutations at the majority of the phosphorylation sites, tau toxicity is abolished. Thus, this suggests that no single phosphorylation site alone contributes to human tau toxicity in flies. The S/P and T/P sites on tau are suggested to co-operate together to mediate neurotoxicity *in vivo* (Steinhilb et al, 2007a; Steinhilb et al, 2007b). In contrast, site-specific mutations in tau have demonstrated that phosphorylation of S396 and S404 might contribute substantially to tau phosphorylation *in vivo* (Steinhilb et al, 2007a). Moreover, it has been confirmed that some *Drosophila* protein kinases are necessary for tau-mediated neurodegeneration, including shaggy and PAR-1, the fly orthologs of GSK3β and MARK, respectively. These kinases are each capable of increasing the toxicity produced by human 2N4R tau and 0N4R tau R406W in the fly eye. Shaggy increased tau phosphorylation of S202, T212, S214, T231, S235, S396 and S404, while PAR-1 also targeted S262. Through analysis of mutant forms of tau, S262 and S396 were found to be the major phosphorylation sites targeted by PAR-1 (Jackson et al, 2002; Nishimura et al, 2004). Furthermore, expression of *Drosophila* cdk5, JNK

kinase and murine PKA enhanced the fly eye toxicity caused by human 0N4R tau and phosphorylation was increased (Steinhilb et al, 2007b).

Other studies demonstrated that human tau toxicity in *Drosophila* is not only determined by phosphorylation or formation of insoluble tau, but also by the alteration of the microtubule-binding properties of tau. As previously mentioned, phosphorylation regulates the microtubule-binding of tau. When phosphorylation is inhibited, tau has an increased affinity for microtubules. A study by Chatterjee et al (2009) showed that human 2N4R tau carrying eleven S/T mutations to alanine resulted in an enhanced eye phenotype upon PAR-1 and shaggy expression. This was explained by the fact that the tau protein was no longer able to detach from microtubules leading to toxicity. Moreover, it has been shown in *Drosophila* larvae that human 0N4R tau carrying fourteen S/T mutations, disrupts axonal transport, reducing the number of motile vesicles within axons as well as their mobility. As seen before, this is due to an increased ability to bind microtubules when tau is dephosphorylated (Talmat-Amar et al, 2011).

In conclusion, using *Drosophila* is likely to provide a sensitive and accurate biosensor in which to evaluate the ability of individual kinases to confer toxicity on tau.

1.11 Aim

My thesis aims to establish a *Drosophila* model of tauopathy to monitor human tau toxicity and to evaluate the role of human kinases during neurodegeneration. Several methods of *Drosophila* transgenesis are analysed to find the optimal conditions to create the animal model. The purpose of this fly model of tauopathy is to gain a greater insight into the role of human kinases in the generation of tau toxicity. Human kinases are tested for their contribution to confer tau toxicity in the fly eye and those generating the most pathogenic tau forms are identified. Selected human kinases relevant to tau pathology are studied, including GSK3β, PKA, CK1δ and DYRK1A. In addition, this work aims to use this *in vivo* model to determine which of the phosphorylation sites on tau is responsible for increased toxicity giving rise to neural degeneration. Ultimately, I aim to establish a *Drosophila* model of tauopathy that is able to provide an optimal tool to identify therapeutic targets and biomarkers of tauopathies such as pathogenic tau kinases and toxic sites of phosphorylation respectively.

CHAPTER 2
Materials and methods

2.1 Genetics

2.1.1 *Drosophila* stocks and husbandry

Flies were maintained on standard cornmeal agar medium (0.8% w/v agar, 2% w/v cornmeal, 8% w/v glucose, 5% w/v Brewer's yeast, 2% v/v ethanol, 0.22% v/v methyl-4-hydroxybenzoate, 0.38% v/v propionic acid) at either 18°C or room temperature. Crosses were maintained at 18°C or 25°C on rich food (0.8% w/v agar, 6% w/v glucose, 3% w/v sucrose, 8% w/v Brewer's yeast, 2% w/v yeast extract, 2% w/v peptone, 1% v/v methyl-4-hydroxybenzoate, 0.6% v/v propionic acid, 0.05% w/v magnesium sulphate, 0.05% w/v calcium chloride). In all cases w^{1118} were used as wild type. Genetic markers and balancer chromosome are described in Lindsley & Zimm (1992) and Flybase (www.flybase.org).

2.1.2 Production of transgenic flies

Transgenic flies were generated by standard embryonic injection and P-element mediated transgenesis or phiC31-mediated transgenesis by BestGene Inc. (Chino Hills, CA, USA) or GenetiVision (Houston, TX, USA). The mini-white (w^+) marker contained in the expression plasmids was used to map the insertion into the chromosomes using standard mating schemes (Greenspan, 2004). Adult males with P[w^+] insertions were selected by eye colour and the insertions were mapped and balanced using a w^- stock with balancers on the second and third chromosomes. The insertions of all transgenic flies produced were confirmed by DNA sequencing.

Name	Genotype	Source
Balancer line	w^{1118}; If/CyO; MKRS,Sb /TM6B,Hu	Bloomington
GFP line	w^{1118}; P{UAS-mCD8::GFP}/CyO	Bloomington
GMR driver lines	w^{1118}; P{GAL4-ninaE.GMR}12 w^{1118}; GMRGAL4,mCD8::GFP/CyO w^{1118}; GMRGAL4,Ubi,GAL80^{TS}/SM6::TM6	Bloomington This study M. Fanto
2N4Rtau#1	w^{1118}; UAS-2N4Rtau$^{III\text{-}1}$/TM6B,Hu w^{1118}; GMRGAL4/CyO; UAS-2N4Rtau$^{III\text{-}1}$/TM6B,Hu	R. Tuxworth This study
2N4Rtau#2	w^{1118}; UAS-2N4Rtau$^{II\text{-}2}$/CyO	R. Tuxworth
2N4Rtau#3	w^{1118}; UAS-2N4Rtau$^{III\text{-}3}$/TM6B,Hu	R. Tuxworth
2N4Rtau#4	w^{1118}; UAS-2N4Rtau$^{III\text{-}4}$/TM6B,Hu	R. Tuxworth
2N4Rtau#5	w^{1118}; UAS-2N4Rtau$^{II\text{-}5}$/CyO	R. Tuxworth
2N4Rtau#51C	w^{1118}; UAS-2N4Rtau$^{II\text{-}51C}$/CyO	R. Tuxworth
2N4Rtau#68A	w^{1118}; UAS-2N4Rtau$^{III\text{-}68A}$/UAS-2N4Rtau$^{III\text{-}68A}$ w^{1118}; GMRGAL4/CyO; UAS-2N4Rtau$^{III\text{-}68A}$/TM6B,Hu	This study
2N4Rtau#68E	w^{1118}; UAS-2N4Rtau$^{III\text{-}68E}$/TM6B,Hu	This study
2N4Rtau#86F	w^{1118}; UAS-2N4Rtau$^{III\text{-}86F}$/UAS-2N4Rtau	This study
2N4Rtau#96E	w^{1118}; UAS-2N4Rtau$^{III\text{-}96E}$/TM6B,Hu	This study
2N4RtauS202A	w^{1118}; UAS-2N4RtauS202A$^{III\text{-}68A}$/TM6B,Hu w^{1118}; GMRGAL4/CyO; UAS-2N4RtauS202A$^{III\text{-}68A}$/TM6B,Hu	This study
2N4RtauT205A	w^{1118}; UAS-2N4RtauT205A$^{III\text{-}68A}$/TM6B,Hu w^{1118}; GMRGAL4/CyO; UAS-2N4RtauT205A$^{III\text{-}68A}$/TM6B,Hu	This study
2N4RtauT212A	w^{1118}; UAS-2N4RtauT212A$^{III\text{-}68A}$/TM6B,Hu w^{1118}; GMRGAL4/CyO; UAS-2N4RtauT212A$^{III\text{-}68A}$/TM6B,Hu	This study
2N4RtauS404A	w^{1118}; UAS-2N4RtauS404A$^{III\text{-}68A}$/TM6B,Hu w^{1118}; GMRGAL4/CyO; UAS-2N4RtauS404A$^{III\text{-}68A}$/TM6B,Hu	This study
MF_0N4RtauR406W	w^{1118}; UAS-0N4RtauR406W/UAS-0N4RtauR406W w^{1118}; GMRGAL4/CyO; UAS-0N4RtauR406W/TM6B,Hu	M. Feany This study
2N4RtauR406W	w^{1118}; UAS-2N4RtauR406W$^{III\text{-}68A}$/TM6B,Hu w^{1118}; GMRGAL4/CyO; UAS-2N4RtauR406W$^{III\text{-}68A}$/TM6B,Hu	This study

Name	Genotype	Source
0N4Rtau	w^{1118}; UAS-0N4Rtau$^{III\text{-}68A}$/TM6B,Hu w^{1118}; GMRGAL4/CyO; UAS-0N4Rtau$^{III\text{-}68A}$/TM6B,Hu	This study
0N4RtauR406W	w^{1118}; UAS-0N4RtauR406W$^{III\text{-}68A}$/TM6B,Hu w^{1118}; GMRGAL4/CyO; UAS-0N4RtauR406W$^{III\text{-}68A}$/TM6B,Hu	This study
Shaggy	w^{1118}; P{UAS-sgg.B}MB5	Bloomington
GSK3β#1	w^{1118}; UAS-GSK3β-Myc/CyO	R. Tuxworth
GSK3β#2	w^{1118}; UAS-GSK3β-Myc/TM6B,Hu	R. Tuxworth
GSK3β#3	w^{1118}; UAS-GSK3β-Myc/CyO	R. Tuxworth
GSK3β#4	w^{1118}; UAS-GSK3β-Myc/TM6B,Hu	R. Tuxworth
PKA	w^{1118}; UAS-PKAcatα/CyO	R. Tuxworth
CK1δ	w^{1118}; UAS-Flag-CK1δΔ317/CyO	R. Tuxworth
DYRK1A	w^{1118}; UAS-Flag-DYRK1A/TM6B,Hu	This study
2N4Rtau + GSK3β	w^{1118}; 2N4Rtau,GSK3β-Myc/TM6B,Hu	This study
GSK3β + CK1δ	w^{1118}; UAS-Flag-CK1δΔ317/CyO; GSK3β-Myc/TM6B,Hu	This study
GSK3β + DYRK1A	w^{1118}; UAS- GSK3β-Myc,Flag-DYRK1A/TM6B,Hu	This study

Table 2.1 *Drosophila* stocks used in this study.

Chromosome	Cytological location	Company
II	51C	BestGene
III	68A	GenetiVision
III	68E	BestGene
III	86F	BestGene
III	96E	BestGene

Table 2.2 Sites used for the phiC31-mediated transgenesis. Chromosomes and cytological sites used to insert human tau transgenes in the *Drosophila* genome.

2.2 Microscopy

2.2.1 Light microscopy for adult eyes

Flies were placed in 1.5 ml tubes and snap-frozen in liquid nitrogen. After thawing, the flies were photographed using an Olympus DP71 camera mounted on a Leica MZ16 stereo microscope using ambient light to minimise glare.

2.2.2 Fly fixation for scanning electron microscopy

Whole flies were immersed in EM fixative solution (Appendix) for 2 hours and rinsed twice with distilled water. Flies were dehydrated by replacing the water with 30%, 50%, 70%, 90% and 100% v/v ethanol solutions, each incubated for 4 to 12 hours at room temperature.

2.2.3 Scanning electron microscopy

Scanning electron microscopy (SEM) was performed in the Centre for Ultrastructural Imaging at King's College London. Samples were fixed and critical point dried as described in Wolff (2000), then sputter-coated in gold and imaged using an FEI Quanta 200F microscope operated at 5 kV in high vacuum mode.

2.3 Histological techniques

2.3.1 Adult eye embedding in epoxy resin

Adult flies were anesthetised, heads were cut and one eye was gently removed using a scalpel. Wounded heads were fixed in 0.1 M sodium phosphate buffer (Appendix) supplemented with 1% v/v glutaraldehyde on ice for 30 minutes, then incubated in a 1:1 solution of OsO_4 (Electron Microscopy Sciences) (Appendix) and 0.2 M sodium phosphate buffer (Appendix) for 2 hours on ice. Heads were dehydrated in ethanol washes (30%, 50%, 70%, 90% and 100%), each incubated for 5 minutes on ice. Heads were infiltrated with propylene oxide (BDH and Prolabo) for 10 minutes at room temperature and incubated overnight in a 1:1 mix of propylene oxide and epoxy resin (Fluka, Sigma). Finally, heads were embedded in the pure epoxy resin and polymerised overnight at 80°C.

2.3.2 Adult eye sectioning

Semi-thin 1 μm eye sections were cut with a Leica UltraCut microtome using a diamond Diatome knife and post-stained with 1% v/v toluidine blue (Sigma). Examination was performed with a Zeiss Axioplan 2 Imaging microscope. Images were captured using a SpotInsight FireWire camera (Diagnostic Instruments).

2.4 Molecular biology

2.4.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using Go Taq DNA Polymerase (Promega) or Phusion High Fidelity DNA Plymerase (Finnzymes). Amplification reactions were carried out in a MWG-Biotech Primus thermal cycler programmed using the following steps: 2 minutes at 94°C, then 30 cycles of 15 seconds at 94°C, 30 seconds at temperature depending on the set of primers used, 1 minute/kb at 72°C and finally stored at 8°C. PCR amplifications were confirmed by standard agarose gel electrophoresis. Finally, DNA products were stored at -20°C.

Name	Primer sequence
UAS2	CTGCAACTACTGAAATCTGCCAAG
UAS3	CTCTGTAGGTAGTTTGTCC
Tau_Fwr	ATGGCTGAGCCCCGCCAGGAG
Tau_Rev	TCACAAACCCTGCTTGGCCAG

Table 2.3 Sequencing primers. The primers UAS2/3 were used to sequence across the multiple cloning site of the pTW (inducible UASt promoter) expression constructs. The primer sequences of Tau_F and Tau_R correspond to the 5' and 3' end of the human tau cDNA sequence respectively.

2.4.2 DNA gel electrophoresis and fragment purification

DNA gel electrophoresis was conducted in TAE buffer (Appendix) using a 1% w/v agarose gel containing 0.003% v/v GelRed (VWR). DNA samples were supplemented with DNA loading buffer (Appendix) and run alongside 3 µl of 1 kb DNA ladder (New England Biolabs) at 100 V constant voltage. DNA bands were visualised under UV light. For cloning purposes, bands were excised from agarose gels and purified using Geneclean Turbo Kit (MP Biomedicals).

2.4.3 Cloning techniques and DNA preps

Standard cloning techniques were used according to Sambrook et al (1989). DNA digestions were performed using restriction enzymes from New England Biolabs following manufacturer's instructions. Linearised DNA vectors were dephosphorylated using Rapid Alkaline Phosphatase (Roche) and insertions were ligated using T4 DNA ligase (Roche). The ligation mixture was transformed into Subcloning Efficiency DH5α chemically competent cells (Invitrogen). Positive colonies were selected using resistance to the appropriate antibiotic on Luria Bertani (LB) agar plates (Appendix). Single colonies were transferred into 2 ml starter cultures of LB (Appendix) containing the appropriate selective antibiotic. DNA was extracted using QIAprep Spin MiniPrep kit (Qiagen) following manufacturer's instructions. Presence of the correct insert was confirmed by PCR using one primer located in the insert and one in the vector. DNA sequencing was performed by Source Bioscience (lifesciences.sourcebioscience.com). The QIAFilter MidiPrep kit (Qiagen) was used for large scales plasmid purification required for sub-cloning, generation of transgenic flies and transfections.

2.4.4 Genomic DNA isolation

Genomic DNA was isolated from single flies snap-frozen in liquid nitrogen in 1.5 ml tubes. Each fly was grounded using a micropestle in DNA prep buffer (Appendix) supplemented with 200 µg/ml proteinase K (Sigma). The samples were incubated at

56°C for at least 30 minutes and at 100°C for 5 minutes, then spun for 1 minute at maximum speed using a bench top centrifuge to pellet debris. The supernatant was transferred to a new tube and stored at -20°C.

2.4.5 Splinkerette PCR

Splinkerette PCR was used to identify genomic DNA region flanking P-element insertions as described in Potter & Luo (2010). Genomic DNA was extracted as described on the Berkeley *Drosophila* Genome Project website (www.fruitfly.org/about/methods/inverse.pcr.html). Thirty flies were homogenised in 400 µl of splinkerette DNA prep buffer (Appendix) supplemented with 200 µg/ml proteinase K (Sigma). Samples were incubated at 65°C for 30 minutes. Eight hundred µl of LiCl/KAc solution (Appendix) were added and the samples were incubated on ice for at least 30 minutes, then spun for 15 minutes at maximum speed using a bench top centrifuge. The supernatant was transferred to a new tube and 600 µl of isopropanol were added. The mixture was spun for 15 minutes at maximum speed using a bench top centrifuge. The pellet was washed with 75% ethanol and air-dried. The DNA was resuspended in 70 µl TE buffer (Appendix) and stored at -20°C. 0.5-1 µg of genomic DNA were digested with BglII (New England Biolabs, UK). The splinkerette oligonucleotides (Splnk_top and Splink_bottom) were annealed at 95ºC for 3 minutes and subsequently ligated to the digested genomic DNA at room temperature for at least 2 hours in a volume of 50 µl using T4 DNA ligase (Roche). The mixture for the first round PCR was set up using 10 µl of ligated DNA. The two sets of primers used were specific for the 5' and 3' ends of PZ-elements respectively: Splnk#1/5'Splnk#1 and Splnk#1/3'Splnk#1. Amplification reactions were carried out in a MWG-Biotech Primus thermal cycler programmed using the following steps: 1.15 minutes at 98°C, then 2 cycles of 20 seconds at 98°C and 15 seconds at 64°C, 30 cycles of 20 seconds at 98°C, 15 seconds at 61°C, 2 minutes at 72°C and finally store at 8°C. The mixture for the second round PCR was set up using 0.5 µl of the previous PCR reaction. The two sets of primers used were specific for the 5' and 3' ends of PZ-elements respectively: Splnk#2/5'Splnk#2 and Splnk#2/3'Splnk#2. Amplification reactions were carried out in a MWG-Biotech Primus thermal cycler programmed using the following steps: 1.15

minutes at 98°C, then 2 cycles of 20 seconds at 98°C and 15 seconds at 58°C, 30 cycles of 20 seconds at 98°C, 15 seconds at 61°C, 2 minutes at 72°C and finally store at 8°C. The resulting DNA bands were purified using Geneclean Turbo Kit (MP Biochemicals) and sequenced by Source Bioscience (www.lifesciences.sourcebioscience.com). (Potter & Luo, 2010)

Name	Primer sequence
Splinkerette Oligonucleotides	
Splnk_top	GATCCCACTAGTGTCGACACCAGTCTCTAATTTTTTTTTTCAAAAAAA
Splnk _bottom	CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTGAATGAGACTGGTGT CGACACTAGTGG
Splinkerette PCR – Round 1	
Splnk #1	CGAAGAGTAACCGTTGCTAGGAGAGACC
5'Splnk #1	ATAGCACACTTCGGCACG
3'Splnk #1	CACTCAGACTCAATACGACAC
Splinkerette PCR – Round 2	
Splnk #2	GTGGCTGAATGAGACTGGTGTCGAC
5'Splnk #2	ATTCGTCCGCACACAACC
3'Splnk #2	GGATGTCTCTTGCCGAC
Sequencing	
5'Splnk _seq	CCTCTCAACAAGCAAACG
3'Splnk _seq	CGGGACCACCTTATG

Table 2.4 Primers used for the splinkerette PCR.

2.4.6 Expression constructs

To make expression constructs, the ORF of the cDNA of interest was amplified by PCR using Platinum Pfx polymerase (Invitrogen) and cloned into pENTR cloning vectors (Invitrogen). The correct insertion was verified by sequencing. Error-free clones were transferred to the expression vectors pAW (actin promoter) or pTW (inducible UASt promoter) according to manufacturer's instructions (Murphy collection of Gateway-equipped expression vectors, supplied by the *Drosophila* Genetic Resource Centre, University of Indiana) using LR Clonase II (Invitrogen). The pUAStattB expression vector allowed phiC31-mediated transgenesis and was a kind gift of the Basler group (Zurich, Switzerland).

Name	Vector	Insert	Tag	Source
pRIT373	pUASt	2N4R tau	-	R. Tuxworth
pRIT376	pUAStattB	2N4R tau	-	R. Tuxworth
pRIT291	pUASt	GSK3β	Myc	R. Tuxworth
pRIT353	pUASt	PKAcatα	-	R. Tuxworth
pRIT293	pUASt	CK1δΔ317	Flag	R. Tuxworth
pGEP12	pUASt	DYRK1A	Flag	This study
pGEP22	pAMW	GSK3β	Myc	This study
pGEP20	pAMW	PKAcatα	Myc	This study
pGEP19	pAFW	CK1δΔ317	Flag	This study
pGEP18	pAFW	DYRK1A	Flag	This study
pGEP31	pUAStattB	2N4R tau R406W	-	This study
pGEP32	pUAStattB	0N4R tau	-	This study
pGEP34	pUAStattB	0N4R tau R406W	-	This study

Table 2.5 Expression constructs used in this study.

2.4.7 Site-direct mutagenesis

Template for site-directed mutagenesis was the full-length ORF of 2N4R human tau cloned into the UASt cassette of the pTW expression vector. Point mutations were generated using the QuikChange Multi site-directed mutagenesis kit (Stratagene) following manufacturer's instructions and then confirmed by sequencing.

Amino acid change	Original codon	Mutated codon	Mutagenic sequence
S202A	TCC	GCC	TACAGCAGCCCCGGCGCCCCAGGCACTCCCGG
T205A	ACT	GCT	CCGGCTCCCCAGGCGCTCCCGGCAGCCGCTCC
T212A	ACC	GCC	GGCAGCCGCTCCCGCGCCCCGTCCCTTCCAAC
S404A	TCT	GCT	GTGTCTGGGGACACGGCTCCACGGCATCTCAG

Table 2.6 Mutagenic primers used on 2N4R human tau. Amino acid change, codon change and primer sequence used for site-specific mutagenesis of four S/T sites on 2N4R human tau. Point mutations are shown in red in each sequence.

2.4.8 RNA extraction from adult heads

RNA was extracted from 5 to 10 adult heads. Heads were placed in a sterile 1.5 ml tube and homogenised in 500 μl of Tri reagent (Sigma) using a micropestle. The lysate was incubated at room temperature for 5 minutes and subsequently 100 μl chloroform (Sigma) were added. After shaking vigorously for 15 seconds, the mixture was incubated for 15 minutes at room temperature and spun at maximum speed for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh sterile tube and 250 μl of isopropanol were added. After vortexing for 10 seconds, the sample was incubated at room temperature for 10 minutes and spun at maximum speed for 10 minutes at 4°C. The pellet was washed 3 times with ethanol and resuspended in 20 μl nuclease-free water (Promega). RNA samples were immediately used for cDNA synthesis and then stored at -80°C. RNA quality controls, including RNA integrity and RNA purity were performed by QStandard (www.qstandard.co.uk).

2.4.9 Complementary DNA synthesis

Complementary DNA (cDNA) samples were synthesised using ImProm-II™ Reverse Transcription System (Promega) following manufacturer's instructions. The reactions were set up using 500 ng of RNA. The cDNA synthesis was carried out in a Termocycler Rotor-Gene 6000 (Corbett Research) programmed using the following steps: 5 minutes at 25°C, 1 hr at 42°C, 15 minutes at 70°C. The cDNA samples were stored at -20°C.

2.4.10 Quantitative PCR

Quantitative PCR (qPCR) was performed by QStandard (www.qstandard.co.uk) using Termocycler Rotor-Gene 6000 (Corbett Research). The cDNA samples were assessed for the transcript levels of the following genes: *Drosophila* actin, *Drosophila* GAPDH, *Drosophila* EIF-4a, mouse CD8a, human tau.

Name	Primer sequence
Actin_Fwr	GTTGGAGAAGTCCTACGAGCT
Actin_Rev	CAAGCCTCCATTCCCAAGAAC
GAPDH_Fwr	CACTACCCACCCACACTCTA
GAPDH_Rev	TCTGAAGTGTCTCACCCCAT
EIF-4a_Fwr	TCAGAAAATGGATGACCGAAATGAG
EIF-4a_Rev	GGCTTCTCGAAACCATAACCG
CD8a_Fwr	AGTCCTTCAGAAAGTGAACTCTAC
CD8a_Rev	TATCACAGGCGAAGTCCAATCC
Tau _Fwr	CTGAGAACCTGAAGCACCAG
Tau_Rev	TTTGAGCCACACTTGGACTG

Table 2.7 Primers used to perform qPCR.

2.4.11 Fly decapitation

Flies were placed in 2 ml screw cap tubes, snap-frozen in liquid nitrogen and decapitated using a FastPrep24-Instrument (MP biomedical) at 6.5 m/s for 20 seconds. Heads were then separated from thoraces and abdomens using a sieve and transferred to a clean 2 ml tube for homogenisation.

2.4.12 Fly head homogenisation

A volume of 100 µl of cold homogenisation buffer (Appendix) at pH 6.8 supplemented with protease and phosphatase inhibitors (Calbiochem) and garnet beads (MP Biomedical) were added to 20 adult heads. Tubes were shaken twice at 6.5 m/s for 20 seconds and then spun for 10 minutes at 20,000 g at 4°C. The supernatant was transferred to a clean tube and stored on ice. A second homogenisation was then performed by adding 100 µl of cold homogenisation buffer at pH 9.2 to the garnet beads and the cellular matter remaining from the first homogenisation. The tubes were then shaken twice at 6.5 m/s for 20 seconds and then spun for 10 minutes at 20,000 g at 4°C. The second supernatant was combined with the first, obtaining a final pH of 8. The lysate was spun for 30 minutes at 20,000 g at 4°C and the supernatant was transferred in a clean tube. Finally the sample was stored at -80°C.

2.4.13 Sarcosyl-solubility protocol

A volume of 60 µl of cold solubility buffer (Appendix) supplemented with protease and phosphatase inhibitors (Calbiochem) and garnet beads (MP Biomedical) were added to 30 heads. Tubes were shaken three times at 6.5 m/s for 20 seconds and then spun for 5 minutes at 1,000 g at 4°C. The supernatant was transferred to a clean tube and stored on ice. The mixture was brought to 1% v/v N-lauroylsarcosinate (Sigma) and incubated for 1 hour at room temperature with shaking. Samples were spun for 1 hour at 100,000 g at 4°C. The sarcosyl-soluble supernatant was transferred to a new tube, while the sarcosyl-insoluble pellet was resuspended in 50 mM Tris-Hcl pH 7.5.

2.4.14 SDS-PAGE

This procedure was carried out according to Laemmli (1970) and Sambrook et al (1989). Protein samples were separated on 10% w/v polyacrylamide gels using a BioRad mini-protean II gel electrophoresis apparatus. Kaleidoscope pre-stained standard markers were obtained from Biorad. Protein samples were mixed in SDS loading buffer (Appendix) and boiled for 3 minutes before loading onto the gel. The samples were run at 90 V constant voltage through the stacking gel and 150 V constant voltage through the separating gel in ultra pure tris-glycine-SDS buffer (National Diagnostics).

2.4.15 Western blotting

Western blotting was performed according to Sambrook et al (1989). After separation on a 10% acrylamide SDS-PAGE, proteins were transferred on nitrocellulose membrane (Biorad) by electrophoretic transfer for 1 hour at 100 V constant voltage in ultra pure tris-glycine buffer (National Diagnostics). The membrane was blocked in 5% w/v non-fat dried milk in TBS (Appendix). Primary and secondary antibodies were individually diluted in 5% w/v non-fat dried milk in TBS-T (Appendix) and incubated with the membrane overnight and for 45 minutes respectively at 4°C. Membranes were washed with TBS every 10 minutes for 1 hour and then scanned by an Odyssey machine (Li-Cor Biosciences) using 169 μm of resolution and 5 arbitrary units of channel intensity. Fluorescent images were analysed by Odyssey Infrared Imaging System 3.0 software (Li-Cor Biosciences) applying a median top/bottom background method. Protein levels were quantified as intensities of the fluorescent signals drawing a box of constant area around the bands of interest. The images were converted to black and white and then exported as TIF files.

Antibody	Epitope	Host species	Source	Dilution
Total human tau	243-441	Rabbit	Dako	1:10,000
PHF1 human tau	pS396/pS404	Mouse	P. Davies	1:5,000
AT8 human tau	pS202/pT205	Mouse	Innogenetics	1:2,000
Tau1 human tau	195-205	Mouse	Chemicon	1:1,000
AT100 human tau	pT212/pS214	Mouse	Innogenetics	1:1,000
AT270 human tau	pT181	Mouse	Innogenetics	1:1,000
Human PKA, α chain	330-350	Rabbit	Santa Cruz	1:200
Human GSK3β-pS9	pS9	Rabbit	Cell Signaling	1:1,000
c-Myc	EQKLISEEDL	Mouse	Santa Cruz	1:1,000
Flag M2	DYKDDDDK	Mouse	Sigma	1:1,000
β-Actin	-	Mouse	Calbiochem	1:5,000
Mouse IgG 680	-	Goat	Invitrogen	1:15,000
Mouse IgM 800	-	Goat	Rockland	1:15,000
Rabbit IgG 800	-	Goat	Rockland	1:15,000

Table 2.8 Primary and secondary antibodies used in this study. Each antibody is described by name, epitope, source and dilution used on western blot. The epitope description includes numbers to define the amino acid position of the epitope or letters to define its sequence.

2.5 Tissue culture

All tissue culture experiments were carried out using *Drosophila* Schneider 2 (S2) cells.

2.5.1 S2 cell maintenance

Cells were maintained in Insect Express Sf9-S2 medium (PAA) supplemented with penicillin-streptomycin (Gibco) and kept in 25 cm^2 tissue culture flasks (Nunc) at 25°C. Cells were passaged by diluting 1:2 and 1:5 when a density between 6-20 x 10^6 cells/ml was reached.

2.5.2 Transfection of S2 cells

Cells were split in 6 well plates so that each well contained 2 x 10^6 cells in a total volume of 2 ml and incubated at 25°C overnight. The medium was substituted with Insect Express Sf9-S2 medium (PAA) without antibiotic. Cells were transfected using Cellfectin II (Invitrogen) following manufacturer's instructions. The total amount of plasmid DNA transfected was 1.5 µg. After 5 hours of incubation at 25°C, the medium was substituted with Insect Express Sf9-S2 medium (PAA) supplemented with penicillin-streptomycin (Gibco). The transfected cells were left to recover at 25°C overnight. When necessary, induction was performed by adding 100 µM of $CuSO_4$ solution to the cells.

2.5.3 Cell lysis

Cells were washed with PBS (Appendix) and harvested in 250 µl of cold lysis buffer (Appendix) supplemented with protease and phosphatase inhibitors (Calbiochem). The cell lysate was spun at maximum speed for 10 minutes at 4°C and the supernatant was stored at -80°C.

2.6 Statistical data analysis

In this study all statistical analyses were performed using the software Prism 4.0 GraphPad. Transcript and protein quantification data were analysed using one way Anova analysis of variance and a parametric post-test ($p<0.05$). The data on photoreceptor counting were analysed using a Pearson's chi-square analysis and a Fisher's exact test.

2.7 Appendix

Name	Formulation
PBS buffer	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2
TBS buffer	50 mM Tris-HCl, 150 mM NaCl, pH 7.6
TBS-T buffer	TBS, 0.1% v/v Tween 20
TAE buffer	40 mM Tris-HCl, 20 mM acetic acid, and 1mM EDTA
TE buffer	10 mM Tris-HCl (pH 8), 1 mM EDTA
6X DNA loading buffer	30% v/v glycerol, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF
LB medium	1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% w/v NaCl (pH 7)
LB agar	LB medium containing 1.5% w/v agar
1 M sodium phosphate buffer	72% v/v 1 M Na_2HPO_4, 28% v/v 1 M NaH_2PO_4
EM fixative solution	50% Na-cacodylate (pH 7.2), 1% v/v glutaraldehyde, 1% v/v formaldehyde, 0.5% v/v Triton X-100
Homogenisation buffer	50 mM Tris-HCl (pH 6.8 or pH 9.2), 0.3 M NaCl, 1% v/v β-mercaptoethanol
Solubility buffer	10 mM Tris pH 7.5, 0.8 M NaCl, 1 mM EGTA pH 8, 10% w/v sucrose
DNA prep buffer	10 mM Tris-HCl (pH 8), 25 mM KCl, 1 mM EDTA, 0.1% v/v Triton X-100
Splinkerette DNA prep buffer	100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 100 mM EDTA, 0.5% v/v sodium dodecyl sulfate
S2 cell lysis buffer	20 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM NaCL, 1 mM EDTA, 0.5% v/v Triton X-100
4x SDS loading buffer	200 mM Tris-HCl (pH 6.8), 6.4% v/v SDS, 5 mM EDTA, 16% v/v glycerol, 0.25% w/v bromophenol blue

Table 2.9 Formulation of solutions and media. All chemicals and reagents used in this study were obtained from Sigma unless otherwise stated.

CHAPTER 3

Human tau expression in the *Drosophila* eye

3.1 Introduction

Tauopathies are a collection of neurodegenerative diseases characterised by the distinctive accumulation of tau aggregates in neurons. The best known of these pathologies is AD, but tauopathies also include FTDP-17, Pick's disease, progressive supranuclear palsy and corticobasal degeneration. Tau aggregates are composed predominantly of insoluble highly phosphorylated tau protein and are likely to be responsible for the neuronal loss (Ballatore et al, 2007; Bouchard & Suchowersky, 2011; Buee et al, 2000; Hanger et al, 2009; Iqbal et al, 2009; Lee et al, 2001). Human tau is a microtubule-associated protein that stabilises microtubules and promotes microtubule polymerisation in neurons. It is involved in regulation of the cytoskeleton and vesicle transport along axons. In adult human brains, alternative splicing generates six different tau isoforms that differ in the number of amino-terminal and carboxyl-terminal domains (Figure 1.1) (Buee et al, 2000; Burbank & Mitchison, 2006; Goedert et al, 1988; Takuma et al, 2003).

Drosophila is as an excellent model system for studying human neurodegenerative diseases (Bonini & Fortini, 2003; Muqit & Feany, 2002; Reiter et al, 2001). In particular, expression of human tau in *Drosophila* results in neurodegeneration that bears many hallmarks of the tauopathies including age-dependency, presence of abnormally phosphorylated tau and in some cases neurofibrillary tangles (Chatterjee et al, 2009; Jackson et al, 2002; Nishimura et al, 2004; Steinhilb et al, 2007a; Steinhilb et al, 2007b; Wittmann et al, 2001). The *Drosophila* eye has been extensively used to investigate neurodegeneration thanks to its richness in neurons and dispensability for fly viability. Moreover, the fly eye provides a rapid readout of degeneration (Bonini & Fortini, 2003; Muqit & Feany, 2002). Expression of human tau in the *Drosophila* eye has been shown to cause loss of the crystalline-like structure of ommatidia characteristic of wild type flies. In particular, the eye degeneration is observable from the disorganisation of the ommatidial arrangement on the external surface of the optic lobe and loss of photoreceptor neurons inside each ommatidium (Chatterjee et al, 2009; Jackson et al, 2002; Nishimura et al, 2004; Steinhilb et al, 2007a; Steinhilb et al, 2007b; Wittmann et al, 2001).

Many successful fly models of tauopathy have been created to investigate the toxicity of human tau. The main differences between these *Drosophila* models are the human tau isoforms studied and the genetic background of the transgenic flies created (Chatterjee et al, 2009; Jackson et al, 2002; Nishimura et al, 2004; Steinhilb et al, 2007a; Steinhilb et al, 2007b; Wittmann et al, 2001). Firstly, human tau-mediated degeneration in flies has been studied using several different tau isoforms, often exhibiting different levels of toxicity. In the majority of cases, the human tau isoform with all four C-terminal repeats that are responsible for the microtubule-binding activity has been used. To evaluate the role of specific sites to the production of tau toxicity, transgenic flies carrying mutated forms of the human tau gene were created. The most common types of point mutations tested are those thought to either inhibit hyperphosphorylation or to be associated with a specific tauopathy in human patients (Chatterjee et al, 2009; Jackson et al, 2002; Steinhilb et al, 2007b). Secondly, differences in genetic background among the fly tauopathy models derive from the method of transgenesis used. The human tau transgene is inserted into the fly genome using standard P-element mediated transgenesis. An early stage *Drosophila* embryo is injected with two vectors, one containing the transgene of interest and the other one encoding for a transposase. The transgene is integrated in the fly germ cells at a random chromosomal site (Rubin & Spradling, 1982; Spradling & Rubin, 1982). P-element mediated transgenesis is a powerful genetic tool in *Drosophila* research, however one of the major drawbacks concerns the genomic position of the transgene insertion. In particular, the chromosomal insertion site of the transgene occurs at random leading to two major consequences. Firstly, there is a risk that the inserted transgene will disrupt essential genes and secondly, the levels of the transgene expression will differ depending on the genomic position. In addition, the insertion site of the transgene cannot be reproduced, meaning that it is impossible to replicate the insertion event of a transgene in a specific chromosomal site.

Previous studies investigating the variability in toxicity mediated by different wild type or mutated isoforms of human tau have used transgenic *Drosophila* carrying various human tau transgenes inserted at random at different genomic positions. Since expression of the transgene can vary due to positional effects, these fly tauopathy models are characterised by a diverse genetic background and by a variable level of tau

expression. No documentation on the phenotypic reproducibility and transgene expression levels in these tauopathy models has been described.

My thesis aims to establish a *Drosophila* tauopathy model to investigate the contribution of individual human kinases and their phosphorylation sites to the generation of toxic forms of human tau. To be able to define their role, it is important to control for the expression level of the transgenes. The aim of this chapter is to characterise the toxicity levels mediated by human tau in several transgenic *Drosophila* lines made via standard P-element mediated transgenesis. In this chapter, I investigated human tau toxicity using the full-length isoform, characterised by two N-terminal inserts (2N) and four repeated regions at the C-terminal (4R) (Buee et al, 2000; Goedert et al, 1988; Takuma et al, 2003). In particular, the reproducibility of the fly eye degeneration caused by the human tau gene was evaluated in fly lines carrying the same human tau transgene inserted in different genomic positions. To define the effects on expression, protein and transcript levels of the transgene were investigated.

3.2 Results

3.2.1 *Drosophila* models of tauopathy created via P-element mediated transgenesis

I utilised a similar animal model for tauopathies as previous investigations, where human tau was expressed in the *Drosophila* eye using GMRGAL4 that drives expression of the transgene in the eye, including the photoreceptor neurons (Brand & Perrimon, 1993; Duffy, 2002). In order to monitor the reproducibility of the phenotypic effect mediated by human tau expression in *Drosophila*, transgenic lines were created via P-element mediated transgenesis carrying the full-length human 2N4R tau transgenes. The same UAS-human 2N4R tau transgene was inserted at random in five different genomic positions. Expression was induced throughout development at 25ºC and flies were collected between 0 to 5 days post-eclosion. The eye phenotypes caused by expression of human tau were examined by scanning electron microscopy. Control flies expressing GMRGAL4 alone were monitored to identify any background phenotype caused by the expression of the driver. Control eyes are characterised by an oval optic lobe and a regular arrangement of ommatidia (Figure 3.1A). In contrast, when human 2N4R tau is expressed in the fly eye (2N4Rtau#1-5), a disruption of the eye architecture is observed. The degenerative phenotype is characterised by an alteration of eye shape caused by loss of tissue, the disarrangement of the regular pattern of ommatidia caused by cell fusion and occasionally the presence of necrotic tissue consistent with cell death (Figure 3.1B-F). Examination of the phenotypes caused by expression of human 2N4R tau reveals different levels of degeneration in lines carrying the same human transgene inserted in different genomic positions. Lines 2N4Rtau#1 and 4 show a weak disruption of ommatidial patterning concentrated in the posterior part of the eye, while the oval eye shape is conserved (Figure 3.1B and E). Line 2N4Rtau#2 is characterised by a more disorganised structure of ommatidia and a mild loss of tissue in the posterior part of the eye compared to control (Figure 3.1C). Line 2N4Rtau#3 exhibits severe eye degeneration characterised by a strong disorganisation of the ommatidial architecture extending to the whole eye surface. In addition, the presence of necrotic ommatidia and substantial loss of tissue are observable (Figure 3.1D). Line 2N4R#5 shows mild to strong eye degeneration

characterised by ommatidial disarrangement and loss of tissue in the anterior and posterior part of the eye (Figure 3.1F).

The individual transgenic lines have a reproducible phenotype, with exception for line 2N4Rtau#5 that over time has shown some variability in the amount of degeneration. For the most part, the human tau expression phenotype was restricted to the eye only. However, when human tau is over-expressed from line 2N4Rtau#3, the flies suffer from a reduced viability. The number of 2N4Rtau#3 flies hatching from a standard cross with the GMRGAL4 driver was approximately half compared to any other 2N4R tau lines (data not shown).

Human tau expression causes eye degeneration in the fly models created in this study as expected. However, a large variation in the level of phenotype is observable among transgenic lines over-expressing the same human 2N4R tau gene inserted in different genomic positions.

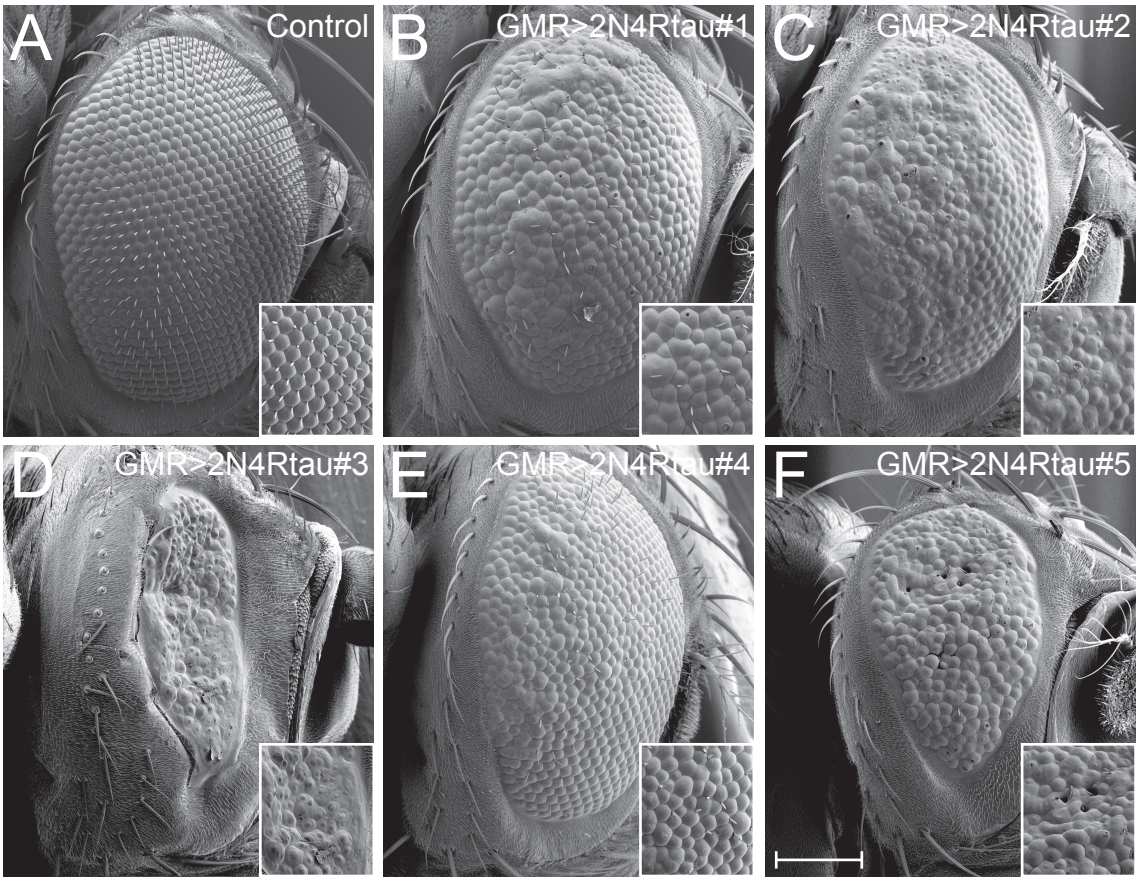

Figure 3.1 Expression of human 2N4R tau in the *Drosophila* eye during development. Scanning electron microscopy pictures of eyes from 0-5 days old flies. (A) Control eyes carrying the eye specific GMRGAL4 driver show a regular array of ommatidia. (B-F) Different degrees of ommatidial disruption result from expression of human 2N4R tau in the eye using GMRGAL4. Each *Drosophila* line has the tau transgene inserted in a different genomic position. Inserts show higher magnification of the central area of the eye. Scale bar: 100 μm (panel) and 25 μm (insert). Dorsal is top and anterior is right.

The transgenic flies over-expressing the same human 2N4R gene created in this study exhibit different levels of degeneration. To identify whether the differences in eye degeneration is a combined result of tau expression and effects on endogenous genes at the insertion sites, the genomic position of the transgene was mapped.
Splikerette PCR is an alternative approach for mapping insertion sites that has been recently developed by Potter and Luo (2010). This technique was originally developed to amplify the genomic DNA between a known restriction site and a target gene, and then it was adapted to map the insertion sites of viral integrating gene traps in the mouse genome. Mapping transposable elements in *Drosophila* using splinkerette PCR is highly efficient. The genomic regions flanking the human tau transgene insertions were sequenced and aligned against the whole *Drosophila* genome.

The locations of the transgenes are shown in figure 3.2A. Lines 2N4Rtau#1, 3 and 4 have the tau transgene inserted on chromosome III, while lines 2N4Rtau#2 and 5 have insertions on chromosome II. In line 2N4Rtau#1, the human tau gene is inserted 300 bp downstream of the first exon of the neurotactin gene (CG9704). The gene neurotactin encodes a surface glycoprotein with homology to serine esterases highly expressed during embryogenesis. It is thought to be a cell adhesion molecule and it is involved in the central nervous system development including axon guidance, axonal fasciculation, heterophilic cell adhesion and axonogenesis (de la Escalera et al, 1990; Hortsch et al, 1990). Line 2N4Rtau#2 has the transgenic insertion in a non-coding region of chromosome II. In line 2N4Rtau#3, the genomic position of human 2N4R tau is 100 bp downstream of the 5' end of the l(3)87Df gene (CG7620) in its second exon. Its molecular function is unknown, however the loss-of-function phenotype affects the longer fly bristles on the notum, known as macrochaetae. Loss of the l(3)87Df gene results in homozygous lethality, in fact transgenic flies homozygous for 2N4Rtau#3 insertion are not viable. In line 2N4Rtau#4, the human 2N4R tau gene is inserted in the central intronic region of the CG7381 gene on chromosome III. The molecular function of CG7381 and the biological processes that rely on the gene are unknown. The human tau transgene insertion in line 2N4Rtau#5 is at the 5' end of the MrgBP gene (CG13746) in its first exon. Although MrgBP is thought to be involved in transcription and neurogenesis, its molecular function is unknown (Jin et al, 2005).

Line 2N4Rtau#3 exhibits the strongest degree of eye degeneration, reduced viability upon human tau expression and it is homozygous lethal, suggesting a potential contribution to the degenerative phenotype due to an effect on the endogenous gene l(3)87Df. Therefore, I investigated whether disruption of the l(3)87Df gene only could contribute to the phenotype of line 2N4Rtau#3.

An independent allele of l(3)87Df was obtained (Bloomington no. 1670) to test whether functional reduction in this gene actively contributed to the phenotype caused by 2N4R tau expression in the fly eye. When the l(3)87Df allele was combined with GMRGAL4, the eye morphology appeared similar to control (Figure 3.2D). One of the tau transgenic lines exhibiting a mild phenotype was crossed with the l(3)87Df allele to see whether the tau-mediated phenotype would be affected. When 2N4R tau was expressed from line 2N4Rtau#1 with GMRGAL4 in the background of the disrupted l(3)87Df gene, the eye phenotype was comparable to line 2N4Rtau#1 expressed in a wild type background (Figure 3.2C and E). This result suggests that the disruption of l(3)87Df is not responsible for the eye degeneration observed in 2N4Rtau#3.

None of the transgenic *Drosophila* lines has the human 2N4R tau transgene inserted in a gene known to be responsible for fly eye development. In particular, line 2N4Rtau#3 with the strongest phenotype does not disrupt an endogenous gene that contributes to the phenotype. All the transgenic lines in the absence of the genetic driver have an eye morphology similar to control (data not shown). Together this evidence suggests that the phenotypic differences in eye degeneration in lines 2N4R#1-5 are not due to effects on endogenous genes disrupted by the insertion.

A

Fly line	Chromosome	Gene	Description
1	III	Neurotactin	- Unkown molecular function - Involved in CNS development, axon guidance, axonal fasciculation, axonogenesis - Phenotypes annotated with: commissure, ventral nerve cord, embryonic CNS, ocellar nerve
2	II	Non-coding region	–
3	III	l(3)87Df	- Unknown molecular function - Phenotype annotated with macrochaeta - Homozygous lethal
4	II	CG7381	- Unknown molecular function - No phenotypic data available
5	III	MrgBP	- Unknown molecular function - Involved in regulation of transcription, DNA-dependent

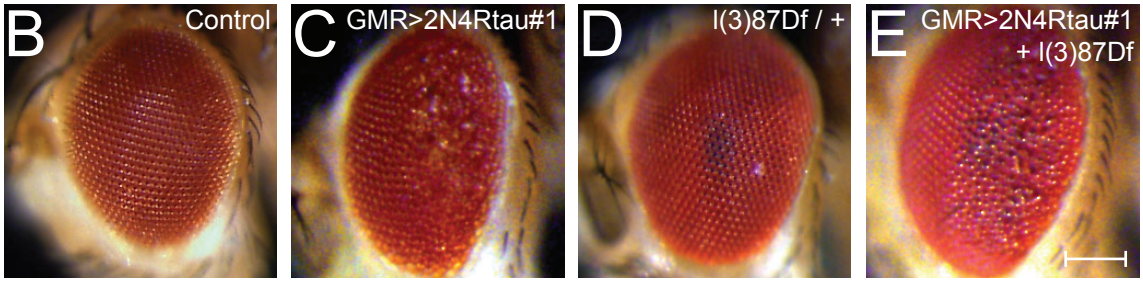

Figure 3.2 Genomic insertion sites of the human tau transgene. (A) Table listing the chromosome where the human tau gene is inserted, the gene nearest the insertion site and a brief description of the gene function (adapted from flybase.com). (B-E) Light micrographs of eyes from 0-5 days old flies. (B) Control eyes carrying the eye specific GMRGAL4 driver show a regular array of ommatidia. (C) Representative example of the disarrangement of ommatidia generated by expression of the human tau gene in the *Drosophila* line 2N4Rtau#1. (D) Flies heterozygous for l(3)87Df do not exhibit any ommatidial disruption. (E) The human tau-mediated eye phenotype in 2N4Rtau#1 is not enhanced by the loss of l(3)87Df. Scale bar: 100 μm. Dashed lines indicate the area of degeneration. Dorsal is top and anterior is left.

To investigate the causes of the variation in eye phenotype upon human 2N4R tau expression from different insertion sites in *Drosophila*, the levels of human tau protein expressed were analysed for each transgenic line.

Human 2N4R tau was expressed throughout development at 25ºC using the eye-specific GMRGAL4 driver. Protein extracts were prepared from heads of flies collected 0 to 5 days post-eclosion (Chapter 2). Expression of human tau was detected by western blotting (Figure 3.3A). An anti-human tau antibody supplied by Dako was used to recognise an epitope on tau independent from its phosphorylation state. The lanes from control flies carrying only the GMRGAL4 driver show no tau expression, suggesting that the anti-human tau antibody is specific and does not cross-react with endogenous *Drosophila* proteins. Following expression driven by GMRGAL4, human tau is detected in all transgenic lines (2N4Rtau#1-5) as a single band at an apparent molecular weight of 65 kDa. The migration of human tau expressed in the flies corresponds to that of recombinant 2N4R tau, suggesting that the phosphorylation state of human tau by endogenous kinases does not lead to a significant change in mobility. Expression levels of human tau were quantified using actin levels to normalise for gel loading. Although the level of tau expression in line 2N4Rtau#3 appears to be much lower compared to the other lines, parametric statistical analysis show that the levels of human tau expression are not significantly different among the transgenic lines (Figure 3.3B). This suggests that the expression levels of human tau protein from the different transgenes do not correlate with the severities of eye degeneration observed in figure 3.1. In particular, despite showing the most severe eye degeneration, line 2N4Rtau#3 has a similar level of human tau expression compared to the other transgenic lines with a milder phenotype.

In conclusion, the levels of human tau protein driven in the tau transgenic lines are similar and do not correlate with the intensity of the degenerative phenotypes, although the individual lines have reproducible levels of tau expression and degeneration.

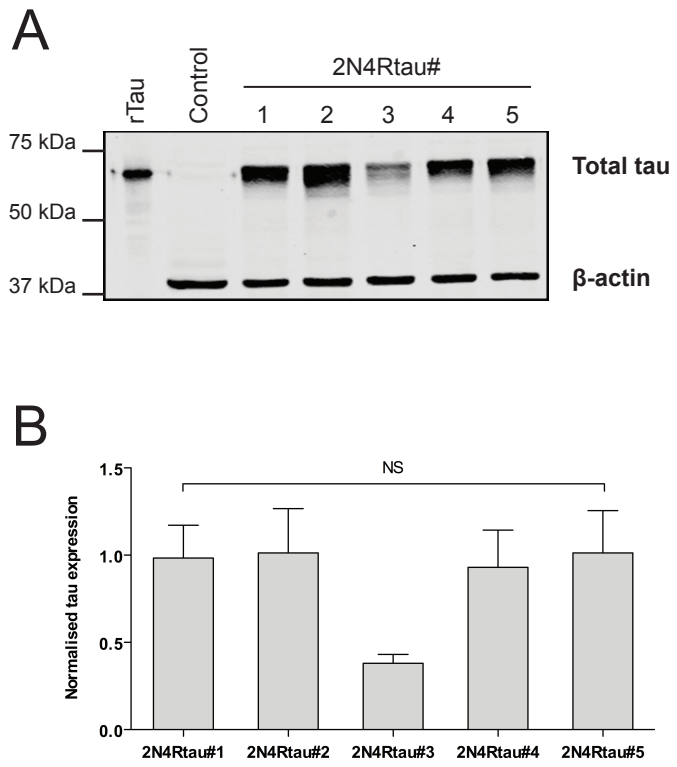

Figure 3.3 Total levels of human tau protein expression in transgenic *Drosophila* lines. (A) Human 2N4R tau and actin expression were detected on western blot of head homogenates from flies expressing the transgene using the eye-specific driver GMRGAL4. Total tau was detected as single band using the phospho-independent antibody Dako (65 kDa) and β-actin was used as loading control (42 kDa). Recombinant human 2N4R tau (rTau) was loaded as positive control for tau and flies carrying GMRGAL4 alone were analysed as negative control. Only the transgenic lines (2N4Rtau#1 to 5) show human tau expression. (B) Protein levels for each transgenic line were normalised to actin. Values correspond to the mean and SEM of 3 replicates of independent experiments. The levels of total tau expression in the transgenic fly lines are non significantly different ($p>0.05$).

The difference in toxicity may be determined by a variation in the solubility of the tau protein. Alterations in tau solubility properties have been shown to be associated with toxic functions of tau (Hirata-Fukae et al, 2009). Sarcosyl fractionation is a widely used method to determine the solubility of human tau. Sarcosyl-insoluble tau has been found to be hyperphosphorylated in AD mouse models (Ishihara et al, 2001; Lewis et al, 2000; Zhukareva et al, 2002). Protocols to isolate PHF-tau from brains of AD patients are based on their insolubility in 1% Sarcosyl (Greenberg & Davies, 1990; Lee et al, 1999). More importantly, it has also been shown that Sarcosyl-soluble and insoluble forms of human tau are produced in *Drosophila* models of tauopathy (Chau et al, 2006). Since the total level of human tau did not correlate with the variations in eye degeneration displayed by the five transgenic *Drosophila* lines created in this study, the Sarcosyl-soluble and insoluble tau levels were analysed.

Protein homogenate obtained from fly heads was resuspended in 1% Sarcosyl and insoluble tau separated by centrifugation at 100,000g (Chapter 2). Human 2N4R tau was expressed throughout development at 25ºC using GMRGAL4. Protein extracts were prepared from heads of flies collected 0 to 5 days post-eclosion. Human tau in the extracts was successfully detected by western blotting using the a phospho-independent tau antibody (Figure 3.4A). Control flies carry the GMRGAL4 driver only and show no tau expression in the soluble or insoluble fraction (data not shown). Following expression using GMRGAL4, human tau is detected in all transgenic lines (2N4Rtau#1-5) in the soluble and insoluble fractions. Human 2N4R tau was represented as a single band at an apparent molecular weight of 65 kDa corresponding to recombinant 2N4R tau. There is no shift in the mobility of either the soluble or insoluble fractions compared to recombinant 2N4R tau, suggesting that the phosphorylation state of human tau by endogenous kinases is not significant in both fractions.

Expression levels of human tau were quantified using actin to normalise for gel loading. As previously seen in figure 3.3, the levels of expression of total human tau are not significantly different between each transgenic line (Figure 3.4B). More importantly, the quantification reveals that the levels of insoluble tau produced are significantly lower than the levels of soluble tau in all transgenic lines. Among each fly line the proportion of tau in the insoluble fraction is similar.

These results show that Sarcosyl-soluble and insoluble human tau forms are produced in the transgenic *Drosophila* models created in this study, however their levels do not correlate with the severity of eye degeneration as seen for the levels of total tau. One of the possible causes of lack of correlation could be related to the loss of eye tissue observed in some of the lines with strongest phenotypes, including lines 2N4Rtau#3 and 5. The normalisation of protein levels in these experiments was conducted using the actin expression of the whole head. There is a possibility that using actin as loading control could not be accurate enough to account for the cell death occurring in the eye only.

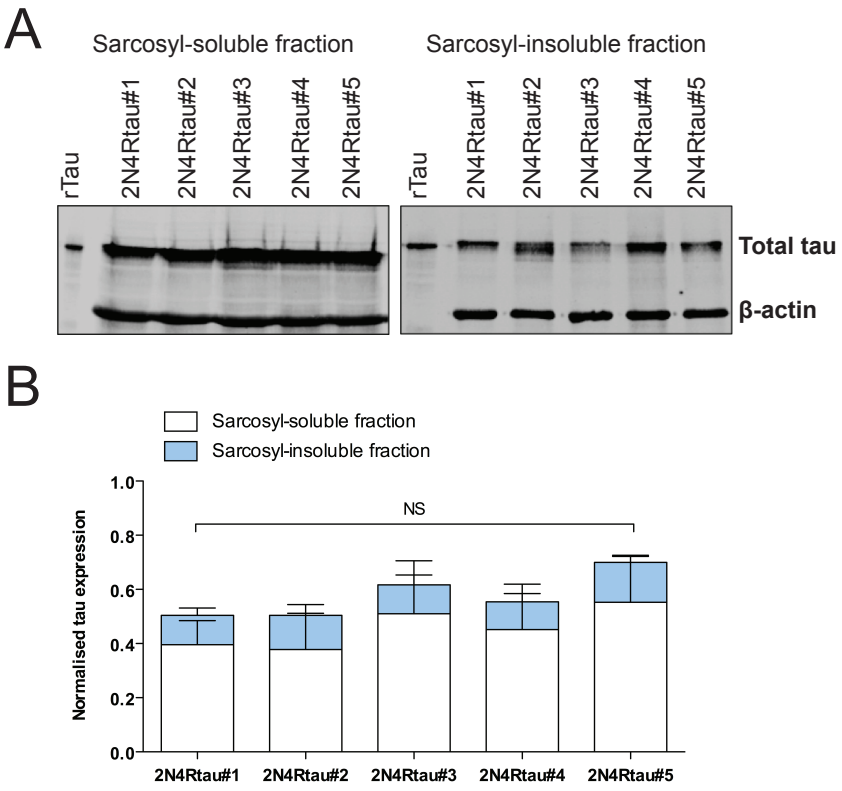

Figure 3.4 Levels of Sarcosyl-soluble and insoluble human tau in transgenic flies generated via P-element mediated transgenesis. (A) Sarcosyl-soluble and insoluble human 2N4R tau and actin expression were detected on western blot of head homogenates from flies expressing the transgene using the eye-specific driver GMRGAL4. Total tau was detected as single band using the phospho-independent antibody Dako (65 kDa) and β-actin was used as loading control (42 kDa). Recombinant human 2N4R tau (rTau) was loaded as positive control for tau and flies carrying GMRGAL4 alone were analysed as negative control. Only the transgenic fly lines (2N4Rtau#1 to 5) show human tau expression in the soluble and insoluble fractions. (B) Soluble (white) and insoluble (blue) tau protein levels for each transgenic were normalised to actin. Values correspond to the mean and SEM of 3 replicates of independent experiments. The amount of insoluble tau is less than that of soluble tau in each transgenic fly line. The levels of soluble and insoluble tau expression are non significantly different between the transgenic fly lines ($p>0.05$).

Previous experiments show that the expression of human 2N4R tau at the protein level does not correlate with the intensity of eye phenotype. Here the levels of human tau protein were normalised against the levels of actin expressed in the whole fly head. Since the expression of human tau was driven only in a subset of cells of the eye, it would be more accurate to use an independent marker expressed within the eye alongside tau for normalisation. Therefore, I included the expression of a reporter gene in the eye together with human tau.

The transcript level of human tau was quantified via qPCR in order to see whether there was a correlation with the variation in intensities of eye phenotype shown in figure 3.1. The experimental design was planned in order to account for loss of eye tissue in the transgenic fly lines, in particular 2N4Rtau#3. Flies were raised at 18ºC and after eclosion they were aged for three days at 25ºC. At lower temperatures the level of GAL4 expression decreases, thus leading to a reduced amount of transgene expression (Duffy, 2002). In this way, transgenic flies expressed a low level of human tau during development at 18ºC to reduce the loss of tissue that occurs when flies were raised at 25ºC (Figure 3.1). After development, the human tau expression was restored raising the temperature to 25ºC. Secondly, the human tau transgene was expressed alongside a reporter gene using the same genetic driver. The spatial and temporal control of the reporter gene is the same as of the human tau transgene. In all *Drosophila* transgenic lines, human 2N4R tau was expressed together with a UAS-CD8::GFP reporter driven by GMRGAL4. The transcript level of human 2N4R tau in each transgenic *Drosophila* line was quantified using actin, GAPDH and the transcriptional factor eIF-4a as reference genes. The copy number of CD8::GFP was used to normalise for the actual number of eye cells expressing human tau. Flies carrying only the eye specific driver GMRGAL4 and reporter UAS-CD8::GFP were used as negative control. There is no expression of human tau in those flies suggesting that the primers designed for qPCR are specific for human tau (Figure 3.5). All transgenic *Drosophila* lines (2N4Rtau#1-5) carry the human tau transgene inserted in different genomic positions and UAS-CD8::GFP expressed using GMRGAL4. As expected, the external ommatidia arrangement of all lines was disrupted by human tau expression compared to control. However, the eye phenotype was overall milder than the one shown in figure 3.1 since flies were raised at lower temperature and thus protein expression was decreased (data

not shown). Upon expression, the transgenic fly lines produce different levels of human tau transcript. To enable a better comparison between the different lines, the transcript levels were expressed in percentage relative to the highest mean. The qPCR result shows that line 2N4Rtau#3 expresses a significantly higher level of human tau transcript compared to all other *Drosophila* tau lines. Lines 2N4Rtau#1, 2, 4 and 5 express an equal amount of human tau transcript that is approximately half of that produced by line 2N4Rtau#3.

These results suggest that the eye degeneration observed in transgenic flies overexpressing human tau correlates with the level of tau transcript expressed. In particular, line 2N4Rtau#3 exhibits the strongest tau-mediated eye degeneration and it is characterised by the highest level of human tau transcript production within the eye compared with the other transgenic lines.

Since my research aim was to examine the role of specific kinases and the sites they phosphorylate on tau, I needed to identify a mechanism that allowed me to control for expression levels of the transgene to allow comparison between wild type and mutant forms of tau. A new approach has been developed to target transgenes to specific insertion sites, thus I examined whether this system could be used in my experiments.

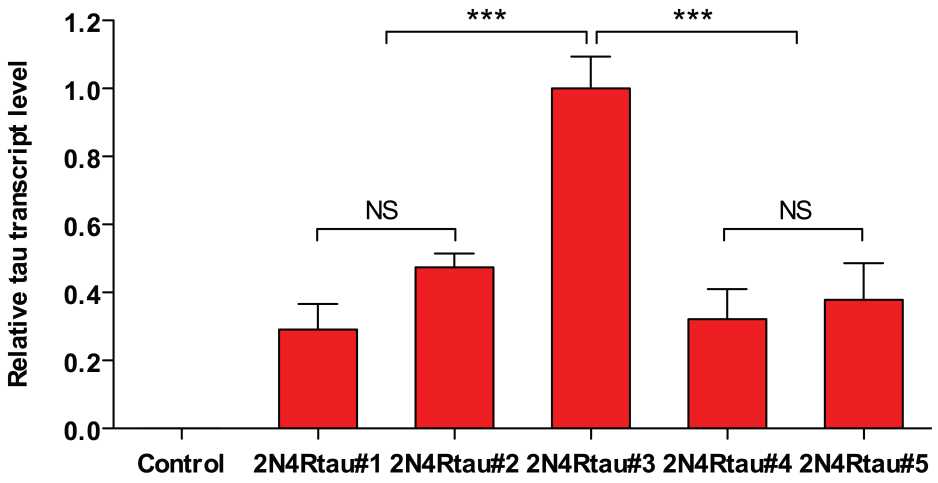

Figure 3.5 Transcript levels of human 2N4R tau are proportional to the level of degeneration caused by expression of the transgene in the *Drosophila* eye. Human 2N4R tau expression was driven together with UAS-CD8::GFP using the eye specific driver GMRGAL4. To enable comparison between samples, the transcript levels were expressed as relative copy number to the highest mean. Tau copy numbers were normalised to 3 reference genes and to UAS-CD8::GFP. Values correspond to the mean and SEM of 3 to 5 replicates from independent experiments. Asterisks indicate statistically significant differences of tau transcript. Control flies carrying the eye specific GMRGAL4,UAS-CD8::GFP driver show no human tau transcript expression. Transgenic flies (2N4Rtau#1 to 5) expressing the 2N4R human tau gene using the eye specific GMRGAL4,UAS-CD8::GFP driver show different tau transcript levels depending on the genomic position of the transgene. All transgenic lines display a highly significant level of tau transcript compared to control. Only line 2N4Rtau#3 shows a significantly higher tau copy number compared to other transgenic lines ($p<0.001$).

3.2.2 *Drosophila* models of tauopathy created via phiC31-mediated transgenesis

An alternative approach has been successfully developed in *Drosophila* to allow the insertion of transgenes in specific genomic loci. This system utilises an integrase derived from phage phiC31 to catalyse a stable recombination between the bacterial attachment site attB and the phage attachment site attP. In *Drosophila*, the phiC31 integrase is used to efficiently integrate attB-containing plasmids into attP landing sites within the fly genome (Bischof et al, 2007; Groth et al, 2004). One of the advantages of using this technique is reproducibility: transgenic *Drosophila* lines can be created carrying transgenes inserted in the same genomic position and thus the same expression levels. This study intends to use this technique to solve the issue related to phenotypic variations of eye degeneration due to positional effects and expression levels of the human tau transgene.

Several transgenic *Drosophila* lines were created carrying the human 2N4R tau transgene inserted in different genomic positions using the phiC31 site-specific integration. The genomic sites used are located on chromosome II and III: 51C, 68A, 68E, 86F and 96E. These cytological positions were chosen due to their availability at the time the transgenic lines where produced. Human tau was over-expressed from these insertion sites throughout fly development at 25ºC using the eye specific driver GMRGAL4. The eye phenotypes caused by expression of human tau were examined using a light microscope. Figure 3.6 shows the eye degeneration phenotype of transgenic fly lines carrying the same human 2N4R tau gene inserted in specific genomic positions. Control flies expressing GMRGAL4 alone were used to monitor the background phenotype caused by the expression of the GAL4 driver. Control eyes are characterised by an oval optic lobe and a regular arrangement of ommatidia (Figure 3.6A). Not all transgenic *Drosophila* lines exhibited a tau-mediated degeneration of the eye when tau expression was driven from the targeted insertions. When 2N4R tau was expressed from insertions 2N4Rtau#51C and 68E, the flies have a regular eye morphology similar to control (Figure 3.6B and D). In contrast, the other fly lines over-expressing human 2N4R tau exhibit a disruption of the eye architecture. In particular, lines 2N4Rtau#68A, 86F and 96E are characterised by a weak disarrangement of the

ommatidial pattern compared to control (Figure 3.6C, E and F). No alteration of eye shape or necrotic tissue is present.

The level of eye degeneration caused by human tau in *Drosophila* eyes is proportional to the transcript level of the transgene as shown above. Therefore, the weak level of eye degeneration shown by 2N4Rtau#68A, 86F and 96E could be explained by a low level of expression of human 2N4R tau. Lines 2N4Rtau#68A and 86F were considered for further investigations. Line 2N4Rtau#96E was discarded because over time it has shown inconsistent eye degeneration.

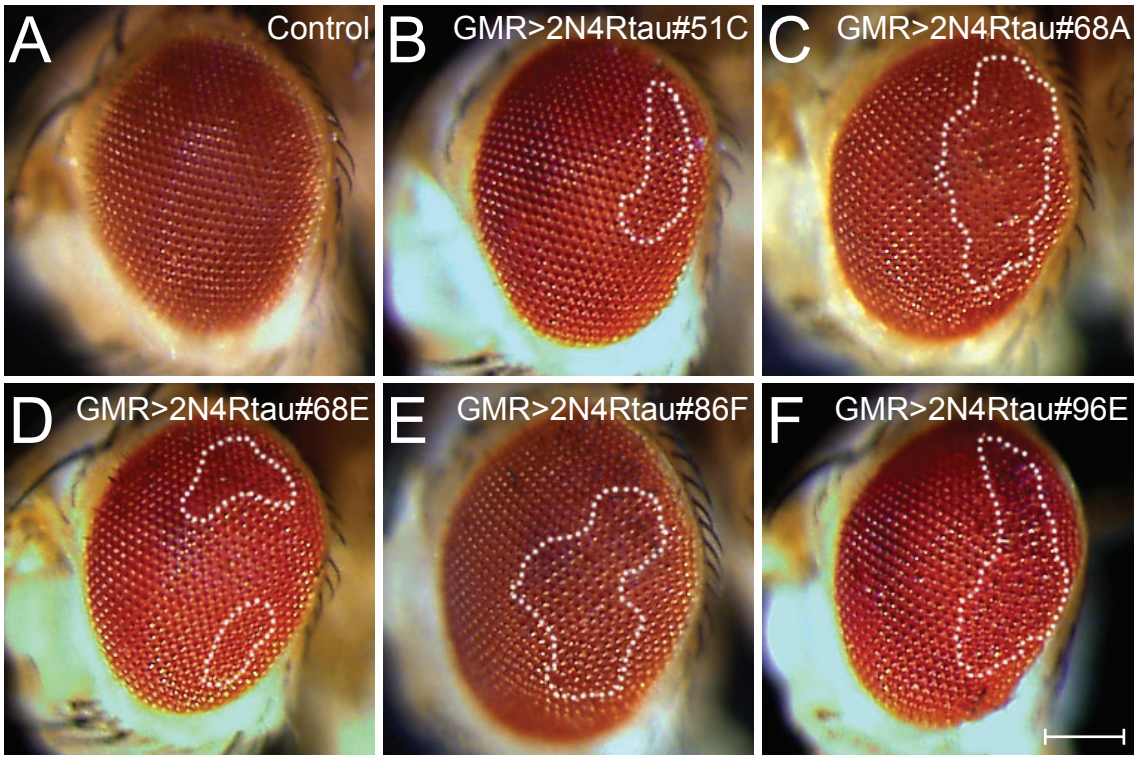

Figure 3.6 Expression of human 2N4R tau from transgene inserted at phiC31-specific sites causes a mild phenotype in the *Drosophila* eye. Light micrographs of eyes from 0-5 days old flies. (A) Control eyes carrying the eye specific GMRGAL4 driver show a regular array of ommatidia. (B-F) The human 2N4R tau transgene was inserted in 5 different genomic positions via phiC31-mediated transgenesis. When human 2N4R tau is inserted in the cytological position 51C (B) or 68E (D), its expression in the eye does not cause significant disarrangement of ommatidia. When the same human transgene is inserted in the cyto-sites 68A (C) or 86F (E) or 96E (F) and expressed in the eye, a weak ommatidial disruption is observed. Scale bar: 100 μm. Dorsal is top and anterior is left.

To examine the reasons for the weaker eye phenotype, the expression level of tau transcript from the lines created using the phiC31-specific integration was examined. The characterisation of the phiC31 transgenic lines included the quantification of the human tau transcript levels via qPCR. The experimental protocol follows that used in the previous experiment conducted using lines 2N4Rtau#1-5 (Figure 3.5).

Human 2N4R tau was driven in the eye by the GMRGAL4 driver together with a UAS-CD8::GFP reporter line. Flies were raised at 18ºC and subsequently aged for three days at 25ºC. In order to be able to compare the transcript levels of lines 2N4R#68A and 86F with the random insertion lines 2N4Rtau#1-5, three cDNA samples produced during the previous experiment were analysed alongside the samples from 2N4Rtau#68A and 86F to monitor reproducibility. The transcript level of human 2N4R tau was quantified in each transgenic *Drosophila* line using actin, GAPDH and the transcriptional factor eIF-4a as reference genes. The copy number of CD8::GFP was used to normalise for the actual number of eye cells expressing human tau. To enable a better comparison between the transgenic lines generated with the phiC31-mediated system and the P-element mediated transgenesis, the transcript levels were expressed in percentage relative to line 2N4Rtau#3 as done for figure 3.5. Flies carrying the GMRGAL4 driver together with the UAS-CD8::GFP reporter were used as negative control and they do not show transcription of human tau (Figure 3.7). Lines 2N4Rtau#68A and 86F produce levels of human tau expression that are not significantly different to one another. Lines 2N4Rtau#68A and 86F express a significantly lower level of human tau transcript compared to 2N4Rtau#3. In particular, the lines generated using the phiC31 site-specific integration produce 80% less human tau transcript compared to 2N4Rtau#3.

This evidence suggests that the weak eye degeneration observed following human tau expression in 2N4Rtau#68A and 86F might be due to the very low level of human tau expressed. Moreover, this study shows that all transgenic lines created with the P-element mediated transgenesis express a significantly increased level of human tau transcript (at least 50%) compared to the lines generated using the phiC31-mediated transgenesis.

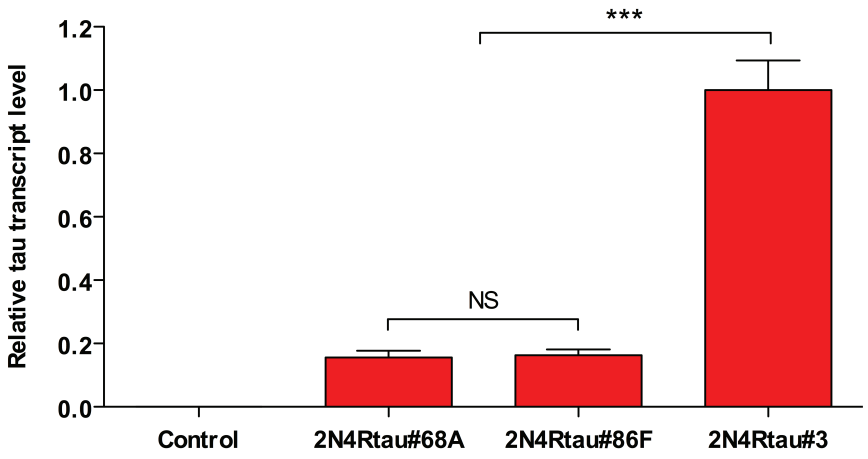

Figure 3.7 *Drosophila* lines generated via phiC31-mediate transgenesis express low transcript levels of human tau in the eye. Human 2N4R tau expression was driven together with UAS-CD8::GFP using the eye specific driver GMRGAL4. To enable comparison between samples, the transcript levels were expressed as relative copy number to the 2N4Rtau#3 mean (Figure 3.5). Tau copy numbers were normalised to 3 reference genes and to UAS-CD8::GFP. Values correspond to the mean and SEM of 5 replicates from independent experiments. Asterisks indicate statistically significant differences of tau transcript. Control flies carrying the eye specific GMRGAL4,UAS-CD8::GFP driver show no human tau transcript expression. The tau transcript levels of the transgenic flies generated via phiC31-mediated transgenesis (2N4Rtau#68A and 86F) are not significantly different (ns). The 2N4Rtau#3 line expresses a four fold greater level of tau transcript compared to 2N4Rtau#68A and 2N4Rtau#86F ($p<0.001$).

The transgenic lines created using the phiC31-specific integration were further characterised at the protein level. Human 2N4R tau was expressed throughout development at 25ºC using the GMRGAL4 driver. Protein extracts were prepared from heads of flies collected 0 to 5 days post-eclosion (Chapter 2). Expression of human tau was successfully detected on a western blot using a phospho-independent tau antibody (Figure 3.8A). Control flies with the GMRGAL4 driver only show no tau expression. Following expression driven by GMRGAL4, human tau is detected in lines 2N4Rtau#68A and 86F as a single band at an apparent molecular weight of 65 kDa corresponding to recombinant tau. Expression levels of human tau were quantified using actin to normalise for protein loading. The levels of expression of human tau are non-significantly different between lines 2N4Rtau#68A and 86F (Figure 3.8B).

This result shows that the levels of human tau protein expressed in the lines created using the phiC31-mediated transgenesis are similar between each other as previously suggested by the quantification of the transcript levels (Figure 3.7).

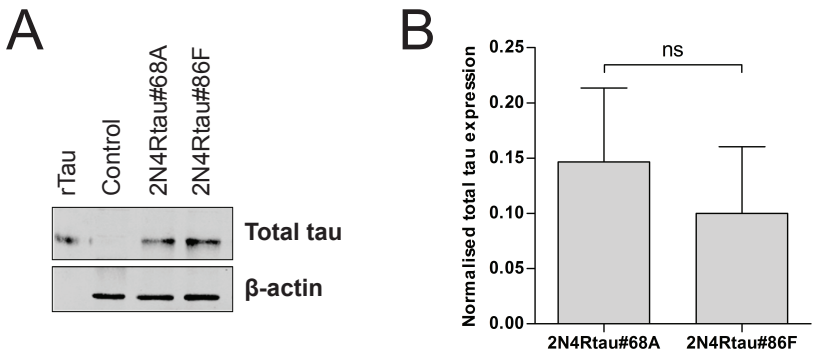

Figure 3.8 Total levels of human tau protein expression in fly lines generated via phiC31-mediated transgenesis. (A) Human 2N4R tau and actin expression were detected on western blot of head homogenates from flies expressing the transgene using the eye-specific driver GMRGAL4. Total tau was detected as single band using the phospho-independent antibody Dako (65 kDa) and β-actin was used as loading control (42 kDa). Recombinant human 2N4R tau (rTau) was loaded as positive control for tau and flies carrying GMRGAL4 alone were analysed as negative control. Only the transgenic lines (2N4Rtau#68A and 86F) show human tau expression. (B) Protein levels for each transgenic line were normalised to actin. Values correspond to the mean and SEM of 3 replicates of independent experiments. The levels of total tau expression in the transgenic fly lines are non significantly different ($p>0.05$).

The transgenic *Drosophila* models previously created in this study produce Sarcosyl-soluble and insoluble forms of human tau as shown in figure 3.4. To investigate whether the transgenic lines created using the phiC31-specific integration produced similar amounts of soluble and insoluble tau, the amount of human tau solubilised by 1% Sarcosyl was assessed (Chapter 2). Human 2N4R tau was expressed throughout development at 25ºC using GMRGAL4. Protein extracts were prepared from heads of flies collected 0 to 5 days post-eclosion and human tau was successfully detected by western blotting using a phospho-independent tau antibody (Figure 3.9A). Control flies carry the GMRGAL4 driver only and show no tau expression in the soluble or insoluble fraction. Following expression using the GMRGAL4 driver, human tau is detected on the blot in lines 2N4Rtau#68A and 86F in the soluble and insoluble fractions. Human 2N4R tau was detected as a single band at an apparent molecular weight of 65 kDa corresponding to recombinant 2N4R tau. As seen for lines 2N4Rtau#1-5 (Figure 3.4), these results confirm that Sarcosyl-soluble and insoluble human tau forms are also produced in lines created using the phiC31-mediated transgenesis. Expression levels of human tau were quantified using actin as normalisation value. The levels of expression of total human tau in lines 2N4R#68A and 86F are not significantly different between each other, consistent with the values in figure 3.7 and 3.8. The quantification reveals that the levels of insoluble tau are significantly lower than the levels of soluble tau. However, the levels of soluble and insoluble tau in lines 2N4R#68A and 86F are similar to that seen for lines 2N4Rtau#1-5 (Figure 3.4).

These results suggest that Sarcosyl-soluble and insoluble tau forms are produced at similar levels in transgenic *Drosophila* created with the site-specific integration system. The amount of soluble tau is greater than insoluble tau as previously observed for the transgenic lines created with P-element mediated transgenesis. Very little insoluble tau is produced from the various *Drosophila* transgenic lines.

The low level of 2N4R tau expression and resultant eye degeneration produced from the GAL4 driven expression of human tau in these lines is advantageous for the identification of enhancers of tau-mediated toxicity. The aim of my thesis is to identify the human kinases generating toxic forms of tau in *Drosophila*, thus an initial low level of tau-mediated toxicity might be useful in determining the nature of the genetic interactions with the human kinases.

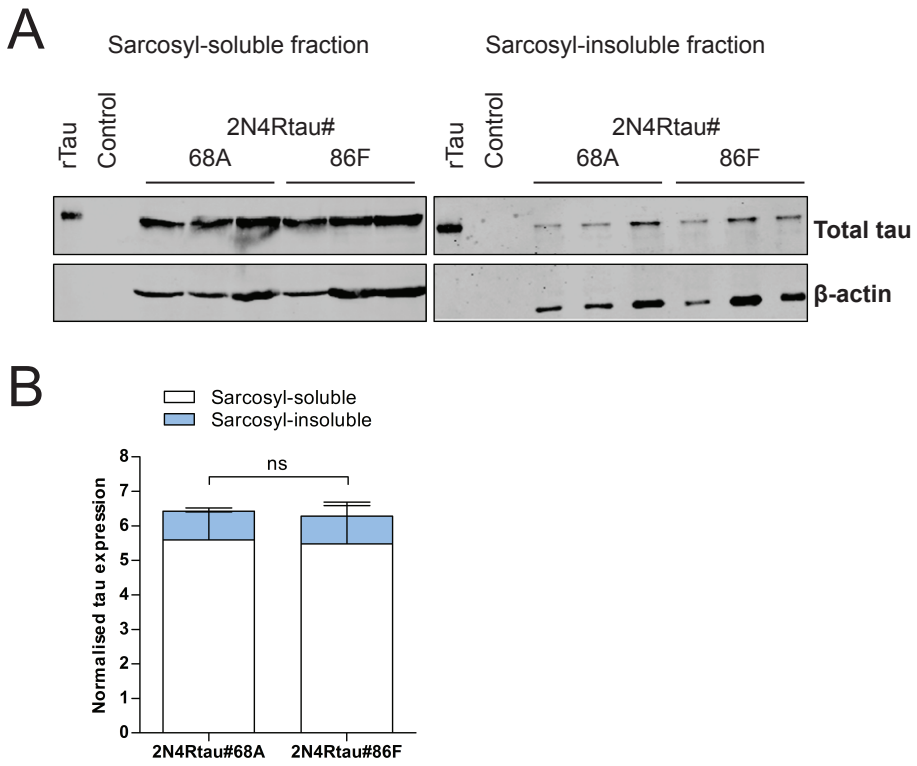

Figure 3.9 Levels of Sarcosyl-soluble and insoluble human tau in fly lines generated via phiC31-mediated transgenesis. (A) Sarcosyl-soluble and insoluble human 2N4R tau and actin expression were detected on western blot of head homogenates from flies expressing the transgene using the eye-specific driver GMRGAL4. Total tau was detected as single band using the phospho-independent antibody Dako (65 kDa) and β-actin was used as loading control (42 kDa). Recombinant human 2N4R tau (rTau) was loaded as positive control for tau and flies carrying GMRGAL4 alone were analysed as negative control. Only the transgenic fly lines (2N4Rtau#68A and 86F) show the human tau protein in the soluble and insoluble fractions. (B) Soluble (white) and insoluble (blue) tau protein levels for each transgenic were normalised to actin. Values correspond to the mean and SEM of 3 replicates of independent experiments. The levels of soluble and insoluble tau expression are non significantly different between the transgenic fly lines ($p>0.05$).

3.2.3 Investigating the photoreceptor degeneration caused by human tau in *Drosophila*

The *Drosophila* eye is a widely used experimental system to identify factors that cause loss of neural tissue. In the previous experiments, human 2N4R tau was expressed in the fly eye throughout development. It is still unclear whether cell death is due to a defect during development of the eye or to the progressive degeneration of differentiated cells, specifically photoreceptor neurons.

To identify if human tau can cause degeneration of differentiated cells, human 2N4R tau was expressed in the fly eye only during adulthood. In order to modulate the temporal expression of human tau, the GMRGAL4 driver was recombined with a GAL4 repressor, $GAL80^{TS}$. The expression of active $GAL80^{TS}$ occurs at 18ºC leading to the sequestration of GAL4 and therefore no transgene is transcribed. When the temperature is increased to 29ºC, $GAL80^{TS}$ is no longer active and transgene expression can occur. Human 2N4R tau was expressed in the fly eye driven by GMRGAL4 in the presence of $GAL80^{TS}$. Embryonic and larval development proceeded at 18ºC in order to prevent human 2N4R tau transcription during development. After eclosion, flies were moved to 29ºC to allow induction of human tau expression. Transgenic *Drosophila* lines were analysed after 1 day or 21 days of human 2N4R tau expression at 29ºC. To investigate the neurodegeneration of photoreceptor neurons, 1 µm sections of fly eyes were produced and membranes were stained with toluidine blue (Chapter 2).

The cells forming the ommatidium are characterised by a concentric organisation. The core of the ommatidium contains eight photoreceptor cells, six of which are organised in an asymmetrical trapezoidal pattern and two lie in the middle. The central photoreceptors develop above one another, therefore only seven photoreceptors are visible on the apical surface. Each photoreceptor is associated with a rhabdomere, a rhodopsin-loaded cell with photosensitive microvilli (Figure 3.10A). The photoreceptor core is surrounded by two cone cells and two primary pigment cells. The outer layer is composed of six secondary pigment cells alternated by three tertiary pigment cells and three bristles. The cells of the outer layer are shared by adjacent ommatidia and together define their hexagonal shape (Ready et al, 1976).

Control flies carry the GMRGAL4 together with $GAL80^{TS}$ alone in order to monitor the background phenotype caused by the expression of the genetic driver. The eye sections of the control flies at 1 day and 21 days of age at 29ºC reveal a very organised eye architecture characterised by a regular pattern of ommatidia each one displaying 7 photoreceptor neurons (Figure 3.10B and F). The transgenic flies analysed include the random insertion line with the highest human tau expression, line 2N4Rtau#3, and the two lines generated using the phiC31 site-specific integration system, 2N4Rtau#68A and 86F. Human 2N4R tau was expressed in the fly eye using the GMRGAL4 driver in the presence of $GAL80^{TS}$. In flies where human tau was induced for 1 day after eclosion, the morphology of all transgenic lines is similar to control, characterised by a regular arrangement of ommatidia displaying 7 photoreceptor neurons (Figure 3.10C-E). When human 2N4R tau expression is induced for 21 days after eclosion, line 2N4Rtau#3 displays a disorganised eye morphology compared to control. In particular, the rhabdomeres are characterised by irregular size and position compared to control (Figure 3.10G). In contrast, after 21 days of human tau induction, lines 2N4Rtau#68A and 86F show a regular arrangement of ommatidia similar to control (Figure 3.10H-I).

In order to measure the degeneration of photoreceptor neurons caused by the expression of human 2N4R tau, the number of photoreceptors per ommatidium was counted in flies over-expressing human tau for 1 day and 21 days after eclosion. The results were plotted on bar graphs showing the percentage of ommatidia displaying the numbers of photoreceptor in each transgenic line (Figure 3.10J and 3.10K). After 1 day of human tau expression, 100% of the ommatidia in the control flies exhibit seven photoreceptors, while 3 to 6% of the ommatidia in lines 2N4Rtau#3, 68A and 86F display six photoreceptors. Following a chi-squared distribution test, all transgenic *Drosophila* lines are characterised by a non-significant difference in photoreceptor number compared to control (Figure 3.10J). After 21 day of human tau expression, between 1 and 2% of the ommatidia display six photoreceptors and 1% displays five photoreceptors in all fly lines. The statistical analysis confirmed that the distribution of photoreceptors in the transgenic lines 2N4Rtau#3, 68A and 86F is not statistically different from the control.

This result suggests that adult onset of human 2N4R tau expression does not lead to photoreceptor neurodegeneration in the transgenic fly lines created in this study. In

addition, it is not possible to detect a difference in eye degeneration that could be associated with the different intensities of tau-mediated phenotypes.

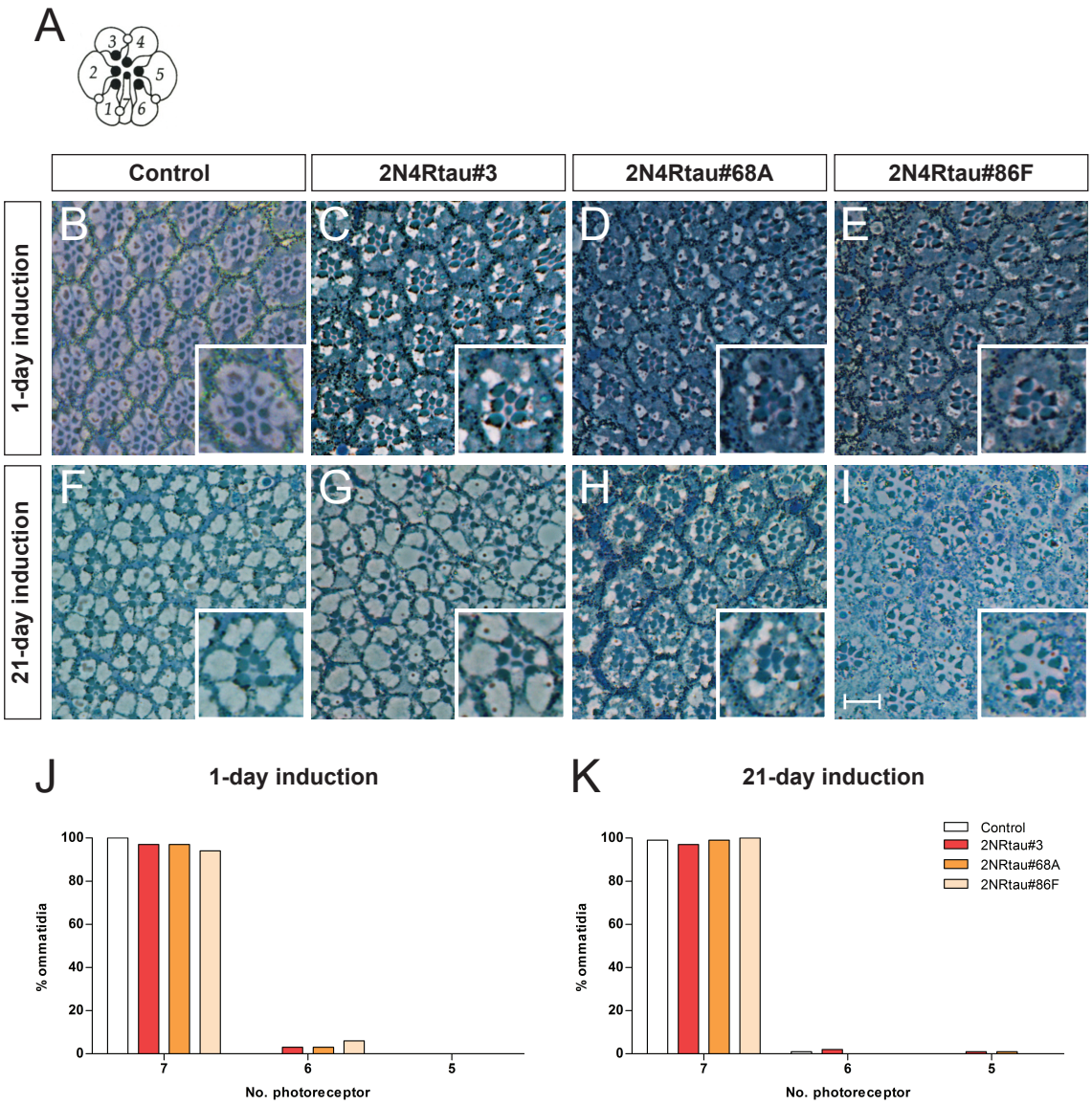

Figure 3.10 Expression of human 2N4R tau during adulthood does not induce photoreceptor degeneration in the fly eye. Human 2N4R tau was overexpressed in the fly eye using the GMRGAL4,GAL80TS inducible driver and induced after eclosion for 1 or 21 days at 29ºC. (A) Schematic structure of an ommadium showing rhabdomeres (1-7) and photoreceptors (black dots). (B-I) Micrographs of eye sections from control flies carrying the GMRGAL4,GAL80TS driver alone and flies expressing human 2N4R tau from transgenes inserted in different genomic positions. Inserts show higher magnification of single representative ommatidia. Scale bar: 10 µm (panel) and 5 µm (insert). (B-E) After 1-day induction of tau expression, the fly lines exhibit an ommatidial structure similar to control. (F-i) After 21-day induction of tau expression, in the 2N4Rtau#3 line a mild disarrangement of the ommatidial structure can be observed compared to 2N4Rtau#68A, 2N4Rtau#86F and control. (J-K) Histograms showing the number of photoreceptors in flies expressing human 2N4R tau inserted in different genomic positions using GMRGAL4,GAL80TS and aged for 1 or 21 days. On average >100 ommatidia were counted coming from 3-5 independent flies. Human tau expression does not cause a significant loss of photoreceptors.

3.3 Discussion

Neurofibrillary tangles containing high levels of hyperphosphorylated tau are one of the main pathological features that contribute to the neurodegenerative process in tauopathies. *Drosophila* is now a well-established model for tauopathies where it is possible to investigate human tau toxicity (Bonini & Fortini, 2003; Muqit & Feany, 2002). Transgenic *Drosophila* models of tauopathy have shown key features of the human disorders including human tau-driven neurotoxicity. In particular, it has already been demonstrated that expression of human tau during the development of the *Drosophila* eye is toxic and causes photoreceptor degeneration (Jackson et al, 2002; Khurana et al, 2010; Shulman & Feany, 2003; Steinhilb et al, 2007a; Steinhilb et al, 2007b; Wittmann et al, 2001). These fly models were created using standard P-element mediated transgenesis that allows the integration of the transgene in random chromosomal sites (Rubin & Spradling, 1982; Spradling & Rubin, 1982). As a consequence, the fly models are characterised by different genetic backgrounds and by a different expression level of the transgene. However, no clear evidence has been shown on the phenotypic reproducibility and transgene expression levels in the fly models created to investigate tauopathies. The aim of my thesis is to characterise the contribution of individual sites in human tau toxicity. To do this I need to establish a model where the transgenes have a uniform level of expression. This chapter investigated the reproducibility of the eye degeneration mediated by human tau in *Drosophila* and characterised the fly models of tauopathy generated in this study by quantifying the transgene expression levels and the neurodegeneration levels.

3.3.1 Variability of tau transgene expression from P-element insertions

Expression of human tau in the *Drosophila* eye produced a degenerative phenotype as previously demonstrated (Jackson et al, 2002; Nishimura et al, 2004; Wittmann et al, 2001). Several transgenic *Drosophila* lines were created via P-element mediated transgenesis carrying the same human 2N4R tau construct. When expressed in the eye photoreceptor neurons, human tau causes several different amounts of degeneration that varies between the transgenic lines carrying the human tau inserted at different genomic

positions at random. The level of tau-mediated degeneration is not reproducible when the same construct is inserted at different sites via P-element mediated transgenesis.

The transgenic flies created in this study differ only by the genomic position of the human 2N4R tau. The variation in the intensity of the eye phenotype upon tau expression could potentially be explained if the chromosomal sites of the transgene interfere with endogenous genes required for eye development. The genomic positions of the human tau transgene were mapped in all transgenic lines. None of the tau insertions disrupts fly genes known to be involved in eye development. Thus, the variation of eye degeneration is not likely due to the alteration or mutagenesis of genes playing a key role in fly eye development. In order to establish the basis of the variation in tau-mediated eye degeneration, a biochemical characterisation of the fly models of tauopathy created in this study was performed. All transgenic lines expressed an equal amount of total 2N4R tau protein in the fly eye, suggesting that the different intensities of eye degeneration is not determined by the levels of expression of human 2N4R tau protein.

The formation of insoluble aggregates of tau is one of the main cellular features of tauopathies. Tau hyperphosphorylation and detachment from microtubules produces species of human tau with altered solubility properties in humans and fly models (Chau et al, 2006; Hirata-Fukae et al, 2009). Wittmann et al, (2001) have shown that in flies expressing human tau neurodegeneration occurs without the formation of neurofibrillary tangles, characteristic of human pathologies. However, tau aggregates have been found in the retina of transgenic *Drosophila* over-expressing human tau, suggesting the possible presence of pre-tangle tau forms (Chatterjee et al, 2009; Jackson et al, 2002). Human tau hyperphosphorylation has been shown to correlate with an alteration of tau protein solubility in flies (Chau et al, 2006; Wittmann et al, 2001). In this investigation, the levels of soluble and insoluble tau were quantified in all transgenic lines expressing 2N4R tau in the fly eye. The majority of the human 2N4R tau expressed was found in a soluble state in each individual transgenic line. However, levels of soluble and insoluble tau did not change when the fly lines where compared to each other. As a conclusion, no direct correlation was found between the levels of insoluble human tau and the severity of toxicity in the eye. Although these results confirm that human tau protein exists in different solubility states in *Drosophila* models of tauopathy as previously

shown (Chau et al, 2006). Finally, qPCR analysis revealed that levels of human tau transcript correlate to the intensities of eye degeneration exhibited by transgenic fly lines carrying the human 2N4R tau transgene inserted in different genomic positions. Potentially, the line exhibiting the strongest eye degeneration was also characterised by the highest tau transcript level. The transcript analysis allowed a more accurate quantification of human tau expression compared to the protein analysis.

This study shows that the human tau-mediated toxicity in the *Drosophila* eye corresponds to the level of transcription expression of the human transgene, but not to the protein level. This discrepancy might be due to two factors. Firstly, the sensitivity between the techniques used is different. The transcript quantification via qPCR is more sensitive than the protein quantification via western blotting and it is not affected by any possible post-translational modification of the protein. Secondly, when analysing the transcript level, a reporter protein was expressed together with human tau in the eye to allow a more precise normalisation. On the other hand, the protein analysis was conducted using actin from the whole head as normalisation factor.

Furthermore, the genomic position of the transgene influences this expression level. The same transgene inserted in different chromosomal sites can be expressed at significantly different levels and ultimately lead to variations in phenotype intensity. These findings agree with recent studies focused on measuring the positional effect of transgenes across different genomic loci in *Drosophila*. Using the GAL4 system Markstein et al (2008) found that the positional effect causes a large variation in the transgene expression from tissue to tissue and therefore there is no single locus that permits optimal gene expression in all tissues. Moreover, the results presented show that the genomic position of the insertion and the expression level of the transgene contribute to determine the intensity of phenotype in a specific tissue in *Drosophila* (Markstein et al, 2008).

3.3.2 Low level of human tau expression from phiC31 insertions

To date, many studies have investigated the effect that single point mutations in human tau might have in modulating tau toxicity in flies induced by endogenous kinases. The mutated tau constructs were inserted via P-element mediated transgenesis at random in the fly genome. The phenotypes observed upon expression of various tau isoforms were compared between the different fly lines in order to compare the toxicity levels. However, the possible phenotype variation caused by the genomic positional effect of the human tau transgene was not considered. One of the aims of this work is to investigate the phenotypic effect of several mutations in human tau, therefore a novel method of transgenesis was used to standardise the transgene expression level and the genetic background of the tauopathies models created.

Recently an alternative approach of transgenesis in *Drosophila* has been developed that allows the integration of transgenes in specific and reproducible genomic loci. This novel method exploits the ability of the phiC31 integrase to mediate recombination between the bacterial and phage attachment sites, attB and attP (Bischof et al, 2007; Groth et al, 2004). A collection of fly lines is available with attP attachment sites located in different non-coding genomic positions. One of the first lines to be described contained the attachment site located in the cytological position 68A flanked by the CG6310 and MocsI genes on the third chromosome (Groth et al, 2004). It has been shown that a luciferase reporter transgene inserted in 68A is optimally regulated using a GAL4 system - it is nearly silent under basal conditions and strongly induced under activating conditions (Markstein et al, 2008). Moreover, since the position of the transgenic insertion within the genome affects its expression and the effects are tissue-dependent, the expression level of the reporter gene inserted in 68A was tested in different tissues, particularly in the larval CNS where it was found to be high compared to other phiC31 insertion sites (Markstein et al, 2008).

In this study, several *Drosophila* lines were created using the phiC31-mediated transgenesis carrying the same human 2N4R tau gene. The chromosomal sites of transgene insertion were chosen in non-coding areas of the *Drosophila* genome and were reproducible. Upon 2N4R tau expression in the fly eye, the phiC31 *Drosophila*

lines exhibit very weak eye degeneration compared to the transgenic lines created with standard methods. Following a biochemical characterisation, the levels of tau transcript were shown to be significantly lower compared to that seen in lines generated by standard transgenesis. Moreover, soluble and insoluble tau isoforms were produced with increased levels of soluble tau over the insoluble form. Additionally, the lines carrying the tau transgene insertion in the cytological locations 51C or 68E do not show eye degeneration compared to control. A reason for this might be due to a particularly low level of tau expressed in the eye, therefore these lines were not considered for my investigation. Taken together these results show that the phiC31-mediated transgenesis can be used to generate fly models of tauopathy that exhibit weak eye degeneration, but more importantly, this transgenesis system overcomes the phenotypic variations due to positional effect of the transgenes. Using this method, different transgenic construct carrying mutated forms of human tau can be inserted in the same genomic loci in different *Drosophila* lines leading to a standardised level of expression and genetic background. Ultimately tau-mediated toxicity in the fly eye would be affected only by the mutation on human tau avoiding any positional effect of the transgene. Since my thesis aims to identify the human kinases that produce toxic forms of tau, another advantage in using this model is the initial low level of degeneration that allows a more easily identification of enhancers of the tau-mediated toxicity.

3.3.3 Human tau does not cause photoreceptor neurodegeneration in adult *Drosophila*

I further examined whether the eye phenotype caused by tau represents neurodegeneration of differentiated neuronal cells, the photoreceptors, or a toxic effect of tau during development. Previous studies show that human tau expression throughout development leads to photoreceptor neurodegeneration as well as progressive vacuolisation and degeneration of cells in the cortex (Chatterjee et al, 2009; Jackson et al, 2002; Nishimura et al, 2004; Wittmann et al, 2001). Here, human 2N4R tau was expressed in the fly eye only during adulthood to see whether the photoreceptor neurons would undergo age-dependent neurodegeneration. Upon ageing the transgenic flies expressing human 2N4R tau did not exhibit photoreceptor loss. However, when

expressed at high levels during development, human tau causes an alteration of the external structure of the eye although the number of photoreceptor does not change when tau is expressed only during adulthood. In conclusion, the tau-mediated phenotypes observed during the previous experiments are not due to neurodegeneration but rather to developmental defects. In the tauopathy model here created the eye phenotype is intended to use as readout for the interaction between human tau and human kinases. Thus, the causes underlying the eye degeneration should not affect the readout of protein interaction.

In conclusion, these data show that in *Drosophila* models of tauopathy, the genomic position and the transcript level of the human tau gene affect the intensity of tau mediated-toxicity in the fly eye. The phiC31-mediated transgenesis of the human tau gene leads to the creation of a more standardised animal model where the level of transgene expression is controlled and the insertion event can be reproduced. The transgenic lines created with the site-specific integration are characterised by a very low level of transgene expression and tau-mediated toxicity. This might be an advantage in the identification of enhancers of the phenotype, for example kinases that might exacerbate the pathogenic role of tau in *Drosophila*.

CHAPTER 4

Human kinase phosphorylation of tau in *Drosophila*

4.1 Introduction

4.1.1 Tau hyperphosphorylation in tauopathies

There is growing evidence that hyperphosphorylation of tau triggers its aggregation into tangles (Alonso et al, 2001; Ballatore et al, 2007; Lauckner et al, 2003). Another possibility is that after aggregation, tau becomes hyperphosphorylated, adopting an altered conformation and thereby inhibiting the action of protein phosphatases. Ultimately, phosphorylation of tau plays a critical role in the development of AD pathogenesis because it alters the microtubule-binding affinity of tau (Amniai et al, 2009; Ballatore et al, 2007; Iqbal et al, 2009). Potential therapeutic strategies against tauopathies have focused on reducing tau phosphorylation through inhibition of specific protein kinases (Lau et al, 2002). This approach aims to reduce tau aggregation and associated neuronal loss hopefully slowing neurodegeneration in patients (Churcher, 2006). Recently, the validity of this approach has been demonstrated by successful *in vivo* experiments using transgenic animals. In fact, it has been shown that tau kinase inhibitors can reduce tau hyperphosphorylation and disease-associated phenotypic defects (Hung et al, 2005; Lewis et al, 2000; Munoz-Montano et al, 1997; Nakashima et al, 2005; Noble et al, 2005). Therefore, it is crucial to identify the full range of protein kinases and phosphatases that regulate tau phosphorylation in order to find the full complement of therapeutic targets.

There is no *in vivo* evidence of the kinases that are specifically involved in the production of pathogenic tau hyperphosphorylation in tauopathies. However, many kinases have been shown to phosphorylate tau *in vitro* (Anderton et al, 2001; Cruz & Tsai, 2004; Feijoo et al, 2005; Hanger et al, 2009; Hanger et al, 2007; Mandelkow et al, 2004). The potentially major protein kinases for pathological phosphorylation of tau include glycogen synthase kinase-3β (GSK3β), cAMP-dependent protein kinase (PKA) and casein kinase 1δ (CK1δ). The phosphorylation sites that are crucial for the physiological and pathological tau role *in vivo* remain unknown. The sequence of tau contains 85 potential phosphorylation sites, including 45 serines, 35 threonines and 5 tyrosines. However, in post-mortem control brains only 17 of them have been found to be phosphorylated. On the other hand, in AD brains recent studies have identified 39

phosphorylated sites via mass spectrometry and 6 via phospho-antibody immunoreactivity (Hanger et al, 2009; Hanger et al, 2007; Lebouvier et al, 2009; Morishima-Kawashima et al, 1995).

GSK3β has been shown to phosphorylate tau both *in vitro* and in cultured cells at epitopes known to be phosphorylated in post-mortem AD brain tissue (Hanger et al, 2007; Hanger et al, 1992; Jicha et al, 1999; Lovestone et al, 1994b). Tau extracted from transgenic mice over-expressing an inducible form of GSK3β has a higher level of phosphorylation than control brain tau. Moreover, transgenic mice exhibiting tangle pathology treated with lithium, an inhibitor of GSK3β, show a reduction of tangle load (Noble et al, 2005). Several pharmaceutical companies now have inhibitors of GSK3β under development as therapeutic agents for AD. However, it is not clear whether inhibition of GSK3β alone would be sufficient to alleviate disease and it is necessary to identify whether further kinases contribute to tau pathology since they may be potential additional therapeutic candidates. *In vitro* phosphorylation studies with purified protein kinases and recombinant tau has shown that GSK3β can phosphorylate at least 26 but not all of the identified tau phosphorylation sites from post-mortem AD brains (Hanger et al, 2009; Hanger et al, 2007).

It has been shown that PKA can phosphorylate tau on up to 19 sites causing a reduction of microtubule assembly *in vitro* (Hanger et al, 2009; Pierre & Nunez, 1983; Scott et al, 1993; Singh et al, 1994). PKA phosphorylation sites reside on the microtubule-binding C-terminal region comprising residues 187-430 (Steiner et al, 1990). (Jicha et al, 1999) demonstrated that PKA is tightly associated with the neurofibrillary pathology, positioning PKA to participate directly in tau hyperphosphorylation. In particular, PKA-dependent phosphorylation of S214 and S409 is increased in PHF-tau compared to control brains and it may be an early step in the conversion of tau in a pathogenic state. Moreover, abnormal phosphorylation of tau by PKA has been suggested to result in a protease-resistant tau population which may contribute to the formation of tau aggregates in tauopathies (Litersky & Johnson, 1992).

CK1 is a family of kinases highly overexpressed in AD brains and mainly found within neurofibrillary lesions in both AD and other tauopathies (Ghoshal et al, 1999; Schwab et al, 2000; Yasojima et al, 2000). They appear as major players in tau aggregation and

filament formation as well as in Aß processing (Flajolet et al, 2007; Hanger et al, 2007; Kuret et al, 1997). CK1δ is a particularly robust marker of granulovacuolar degeneration bodies and the major source of basal phosphorylation activity on S202, T205, S396 and S404 of tau *in vitro* (Ghoshal et al, 1999; Hanger et al, 2007; Li et al, 2004). Expression levels of CK1 has been reported to be elevated more than 20 fold in post-mortem AD brain (Yasojima et al, 2000). Upon CK1δ phosphorylation of tau, a reduction in tau bound to detergent-insoluble microtubules is present, suggesting that CK1δ phosphorylates tau at sites that modulate tau binding to microtubules (Li et al, 2004). These observations lead to the hypothesis that CK1 enzymes regulate tau phosphorylation and that their up-regulation in level or activity contributes to tau hyperphosphorylation in disease. CK1δ can phosphorylate tau on at least 22 sites; a combination of GSK3β and CK1δ can phosphorylate more than three-quarters of the sites that are known to be phosphorylated in PHF-tau (Hanger et al, 2009; Hanger et al, 2007).

Recently, novel kinases have been suggested to be involved in the pathogenic production of toxic forms of hyperphosphorylated tau protein, including DYRK1A (Liu et al, 2008; Wegiel et al, 2008). This kinase has been shown to contribute to the aberrant brain development underlying mental retardation in DS. Interestingly, the DYRK1A locus is localised on human chromosome 21, within the DS critical region (Marti et al, 2003). In addition, it has been shown that early-onset AD is a feature of DS patients (Altafaj et al, 2001; Ryoo et al, 2007). Both DS and AD have common pathological hallmarks such as amyloid plaques and NFTs. Higher levels of DYRK1A have been found in DS brains and its presence has been detected in NFTs from patients with sporadic AD (Liu et al, 2008; Wegiel et al, 2008). Moreover, DYRK1A is able to phosphorylate tau *in vivo* mainly in its proline-rich region. The known phosphorylation sites on tau *in vitro* are S199, S202, T205, T212 and S404, which may be important in the loss of microtubule assembly and in the formation of NFTs (Hanger et al, 2009; Liu et al, 2008; Ryoo et al, 2007). Thus, DYRK1A could be a kinase able to generate toxic forms of tau and maybe a novel potential target for the treatment of the tauopathy in both DS and AD patients.

Recent *in vitro* studies have suggested that multiple kinases are likely to be involved in tau phosphorylation and that specific kinases may prime tau for phosphorylation by

others, inducing a kinase cascade. For example, CK1δ, DYRK1A and cdk5 have been suggested to prime tau for subsequent phosphorylation by GSK3β (Li et al, 2006; Woods et al, 2001). Moreover, there is evidence suggesting that phosphorylation of T212 by DYRK1A promotes GSK3β-mediated phosphorylation of tau (Woods et al, 2001). Therefore, targeting the inhibition of a priming kinase may be another promising therapeutic strategy to reduce the overall level of tau phosphorylation.

4.1.2 Tau hyperphosphorylation in *Drosophila* models of tauopathy

Most work investigating the phosphorylation of tau has been performed *in vitro* with purified proteins; *in vivo* validation of these findings is required. Therefore, it is essential to understand the importance of the candidate kinases for tau toxicity and their activity on the predicted sites *in vivo*. *Drosophila* has emerged as an excellent model system for studying human neurodegenerative disease (Reiter et al, 2001). Previous investigations used the fly models of tauopathy to identify endogenous *Drosophila* genes that can modify human tau toxicity. It has been shown that *Drosophila* endogenous kinases phosphorylate tau and possibly have a major role in generation toxic forms of the tau protein in this model (Blard et al, 2006; Chatterjee et al, 2009; Chau et al, 2006; Jackson et al, 2002; Nishimura et al, 2004; Shulman & Feany, 2003; Steinhilb et al, 2007a; Steinhilb et al, 2007b; Wittmann et al, 2001). It has been confirmed that the *Drosophila* GSK3β (shaggy) and MARK (PAR-1) protein kinases are necessary for tau-mediated neurodegeneration in flies (Jackson et al, 2002; Mudher et al, 2004; Nishimura et al, 2004). Additional genetic screens have also identified further endogenous *Drosophila* genes, primarily kinases and phosphatases, which promote or inhibit human tau toxicity (Shulman & Feany, 2003; Steinhilb et al, 2007b). It is likely therefore that *Drosophila* provides an accurate biosensor to evaluate the ability of individual kinases to confer toxicity on tau.

The aim of this chapter is to characterise whether these human kinases can produce toxic forms of human tau *in vivo*. Previous studies using *Drosophila* focused on the role of endogenous *Drosophila* kinases in human tau phosphorylation, this investigation proposes to use the fly model of tauopathy to evaluate the role of specific human

kinases in producing toxic forms of tau. Human tau together with human kinases were expressed together in the *Drosophila* eye to identify which kinases increase tau toxicity. This *in vivo* model allows us to rapidly manipulate and test the interaction between human tau and human kinases. The selected human kinases described above that are relevant to tau pathology are studied, including GSK3β, PKA, CK1δ and DYRK1A. Ultimately, I hope to determine which of the selected human kinases are responsible for production of toxic forms of tau and would make the most promising candidates for therapeutic targets. To gain a greater insight into the role of human kinases in the generation of tau toxicity, this *in vivo* system will also be used to determine the sites on tau that are phosphorylated to give rise to more toxic forms of tau that may cause neural degeneration. The identification of pathogenic phosphorylation sites might also contribute to the detection of potential biomarkers. This is achieved by mutating key phosphorylation sites within human tau and by investigating whether this modifies the ability of the kinases to generate toxic forms of tau.

4.2 Results

4.2.1 Human kinases in *Drosophila*

To examine whether active human kinases could be expressed in *Drosophila* cells, S2 cells were transfected with 2N4R tau and either GSK3β, PKA, CK1δ or DYRK1A. The S2 cell line is the most widely used *Drosophila* cell line and it was originally derived from late embryonic stage *Drosophila* embryos as a spontaneously immortalised, non-clonal cell line. These cells exhibit mesodermal characteristics and their cellular behaviours and gene expression suggest that they are derived from haemocytes (Schneider, 1972).

Expression of human 2N4R tau was controlled by an inducible UAS-GAL4 system. Cells were transfected with two plasmids, one containing the UAS-tau sequence and the other one containing an inducible GAL4 construct. GAL4 expression was controlled by a metallothionein promoter, which can be induced by adding copper sulphate. Human kinase expression was driven by a constitutively active actin promoter. In order to easily detect their expression on western blots, the kinases carried a tag at the 3'end of their cDNA sequences. The full-length human GSK3β sequence was tagged with the myc epitope. The human CK1δ construct encodes a constitutively active truncated protein, which is tagged at the C-terminus with a flag epitope. This truncation at amino acid 317 has been shown to increase CK1δ activity (Graves & Roach, 1995; Longenecker et al, 1996). Similarly, expression of only the α-catalytic domain of human PKA has also been shown to be more active (Skalhegg & Tasken, 2000). This human PKA construct carried a myc epitope tag at the 3'end of its sequence. Finally, rat DYRK1A was tagged at the N-terminus with a flag epitope. Rat DYRK1A is characterised by extremely high sequence similarity with the human orthologue, differing only by three amino acids.
S2 cells were transfected with human tau and a single kinase. Human 2N4R tau expression was induced for five hours while kinases were constitutively expressed (Chapter 2). Cells transfected with an empty vector were used as negative control. Expression of human tau and kinases was successfully detected by western blotting (Figure 4.1A). The anti-human tau antibody supplied by Dako recognises an epitope on tau independent of its phosphorylation state. Control cells carrying an empty vector

show no tau expression, suggesting that the anti-human tau antibody is specific and does not cross-react with the *Drosophila* proteins. Following five-hour expression, human tau is detected on the blot as multiple bands at an apparent molecular weight ranging from 60 kDa to 65 kDa. This might be due to different levels of basal phosphorylation by endogenous kinases leading to the production of stable human tau species characterised by different mobility on western blot. The expression of human and rat kinases were detected using anti-myc and anti-flag antibodies. All kinases ran on western blot at the expected molecular weight: PKA 40 kDa, CK1δ 37 kDa, DYRK1A 100 kDa and GSK3β 60 kDa. Actin expression was used to normalise for gel loading. When human 2N4R tau was co-transfected together with human CK1δ or rat DYRK1A, the pattern of tau bands did not change compared to human tau transfected alone. However, some differences in the tau bands were observed when PKA or GSK3β were co-transfected. Co-expression of human PKA enriched the production of tau forms with high molecular weight. Co-expression of GSK3β led to a more dramatic shift in mobility of human tau. The shifts observed are probably due to phosphorylation of human tau by human PKA or GSK3β in *Drosophila* cells (Figure 4.1A).

In order to monitor the sites of human tau phosphorylated by the selected human kinases, we aimed to perform mass spectrometry on human tau extracted from S2 cells. This technique would allow us to map all the phosphorylated sites present on human tau before and after co-transfection with human kinases. Unfortunately, we were not able to extract enough protein for the mass spectrometry analysis (data not shown). This might be due to the fact that the construct for human tau does not carry a tag, which might have facilitated the purification of the protein. As a consequence, the protein extract derived from S2 cells transfected with human tau and kinases were analysed using phospho-specific antibodies for human tau (Figure 4.1B-F). The phospho-specific antibodies chosen are widely used to study pathogenic tau phosphorylation in tauopathies and they recognise different phosphorylation sites: PHF1 (pS396 and pS404), AT8 (pS202 and pT205), AT270 (pT181), AT100 (pS212 and pS214) and Tau1 (dephosphorylation at Y197-S208) (Goedert et al, 1994; Goedert et al, 1995b; Otvos et al, 1994; Szendrei et al, 1993). Control cells carrying an empty vector show no phospho tau expression, suggesting that all anti-phospho human tau antibodies are very specific and do not cross-react with endogenous *Drosophila* proteins. Human 2N4R tau showed a basal level of phosphorylation of the PHF1 epitope that is significantly

increased only when human GSK3β is expressed (Figure 4.1B). Moreover, the PHF1 antibody detected specifically the higher molecular bands of human tau in all samples, suggesting that the multiple bands representing human tau arise from a differential phosphorylation state of the protein. However, phosphorylation of the AT8 epitope on human tau is very specific and occurs on the higher molecular weight species of tau only when GSK3β is co-expressed (Figure 4.1C). The AT270 antibody reveals a basal level of phosphorylation at its epitope on human tau generated by endogenous kinases but a shift occurs to the tau forms only when GSK3β is co-expressed, similar to the result obtained with the PHF1 antibody (Figure 4.1D). No detection of the AT100 epitope was seen (Figure 4.1E). The Tau1 antibody recognises tau only when S195, S198, S199, S202 and T205 are dephosphorylated. A lower level of dephosphorylation was shown by the highest molecular species of tau when GSK3β is expressed (Figure 4.1F). Together these results lead to the conclusion that human GSK3β is the only human kinase that significantly phosphorylates human tau in *Drosophila* cells at the specific sites tested. It is possible that human PKA, CK1δ and rat DYRK1A phosphorylate tau on other epitopes not analysed with this experiment.

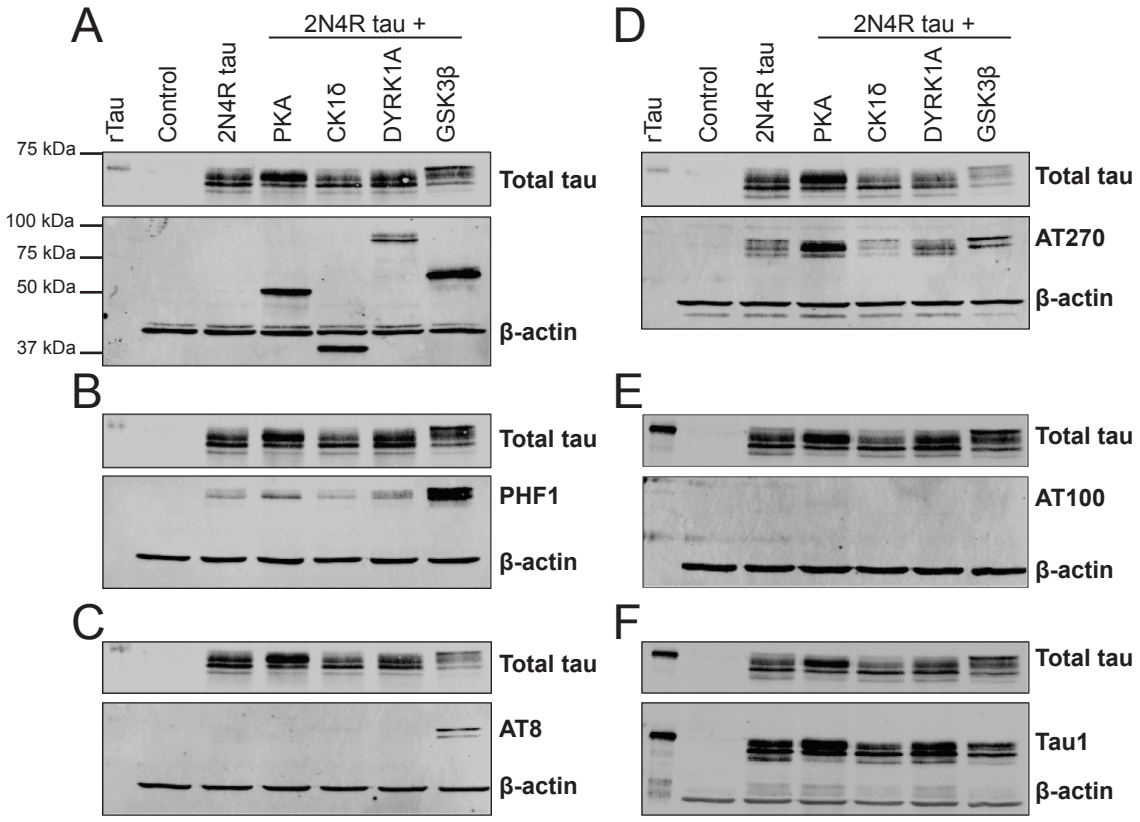

Figure 4.1 Human GSK3β phosphorylates human tau in *Drosophila* S2 cells. After transfection, human 2N4R tau, kinase and actin expression were detected on western blot of S2 cell lysates. Recombinant human 2N4R tau (rTau) was loaded as positive control for tau and S2 cells transfected with an empty vector were used as negative control. Total tau was detected as multiple bands (60-70 kDa) using the phospho-independent antibody Dako. β-actin was used as loading control (42 kDa). (A) Kinases were detected using anti-flag and anti-myc antibodies: PKA (50 kDa), CK1δ (37 kDa), DYRK1A (95 kDa), GSK3β (60 kDa). (B-F) Phosphorylated human tau was detected using phospho-specific antibodies (B) PHF1 for pS396 and pS404, (C) AT8 for pS202 and pT205, (D) AT270 for pT181, (E) AT100 for pT212 and pS214 and (F) the dephospho-dependent antibody Tau1 (S198-S208). Human 2N4R tau alone shows a basal phosphorylation level at PHF1 and AT270 epitopes. Co-transfection of human GSK3β causes a mobility shift of human tau and increased phosphorylation is detected with PHF1, AT8 and AT270.

I aimed to identify whether any of the chosen human kinases could increase the toxicity of the human tau beyond that generated by endogenous kinases. I tested this by expressing specific human kinases with the human tau in the *Drosophila* eye. Many studies demonstrated that human tau-mediated degeneration is enhanced by overexpression of *Drosophila* shaggy (Chatterjee et al, 2009; Jackson et al, 2002; Nishimura et al, 2004). Shaggy is the fly orthologue of GSK3β and it is involved in many processes, including notch and wingless signalling pathways (Cadigan & Nusse, 1997; Foltz et al, 2002). In order to see whether this result could be reproduced using the phiC31 transgenic tau line created in this study, the phenotype of flies co-expressing human 2N4R tau and *Drosophila* shaggy were observed. The transgenic tau line chosen for the experiment was line 2N4Rtau#68A with the human 2N4R tau gene inserted via phiC31-specific transgenesis in position 68A.

Human 2N4R tau and *Drosophila* shaggy was expressed in the fly eye using the GMRGAL4 driver. Gene expression was induced throughout development at 25ºC and flies were collected between 0 to 5 days post-eclosion. The eye phenotypes were examined by light microscopy (Figure 4.2). Control flies express GMRGAL4 alone in order to monitor the background phenotype caused by the expression of the genetic driver. Control eyes are characterised by an oval optic lobe and a regular arrangement of ommatidia (Figure 4.2A). In contrast, when either human 2N4R tau or *Drosophila* shaggy is expressed in the eye, a disruption of the eye architecture is present. Line 2N4Rtau#68A is characterised by a weak disarrangement of the ommatidial pattern compared to control, but no alteration of eye shape or necrotic tissue is present (Figure 4.2B). Over-expression of endogenous shaggy leads to a disruption of the central ommatidia arrangement (Figure 4.2C), as previous studies reported (Chatterjee et al, 2009; Jackson et al, 2002; Nishimura et al, 2004). When human 2N4R tau is co-expressed together with shaggy, an enhancement of eye degeneration is observed (Figure 4.2D). The area of the eye showing ommatidial disarrangement is larger compared to that seen when human tau and shaggy are expressed alone. Moreover, depigmentation is observable in the central area of the eye due to the degeneration of the pigmented cells, part of each ommatidium. Examination of the phenotypes caused in the eye by expression of human 2N4R tau and *Drosophila* shaggy reveals an enhancement of eye degeneration compared to human tau and shaggy expressed alone. Thus, the human tau-mediated degeneration observed in the fly model of tauopathy

created in this study can be enhanced by over-expression of a kinase, in this case the endogenous shaggy. It is possible that this phenotype is due to an addictive developmental toxicity of human tau and shaggy, however the intensity of eye degeneration correlates with previous studies that demonstrate an enhancement of tau toxicity upon shaggy over-expression (Chatterjee et al, 2009; Jackson et al, 2002; Nishimura et al, 2004; Steinhilb et al, 2007b). Line 2N4Rtau#68A can reproduce results previously seen with other fly models of tauopathy and therefore it can be used for further investigations using human kinases.

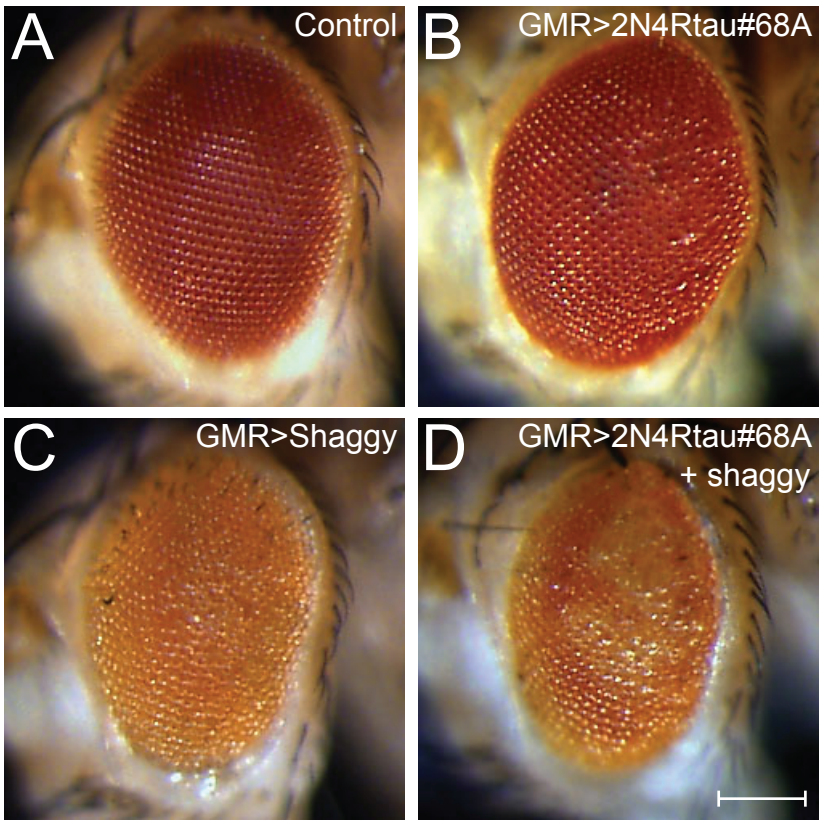

Figure 4.2 Human tau-mediated degeneration is enhanced by co-expression of shaggy in the *Drosophila* eye. Light micrographs of eyes from 0-5 days old flies. (A) Control eyes carrying the eye specific GMRGAL4 driver show a regular array of ommatidia. (B) Expression of human tau from 2N4Rtau#68A causes weak ommatidial disruption. (C) A weak disarrangement of ommatidia is also observed when Drosophila shaggy is expressed. (D) Co-expression of human 2N4R tau and Drosophila shaggy enhances the degenerative phenotype in the eye causing depigmentation and increased roughness. Scale bar: 100 µm. Dorsal is top and anterior is left.

The specific human kinases responsible for the hyperphosphorylation of tau in AD brains are still not known. I used the 2N4Rtau#68A line to test the ability of specific human kinases to increase toxicity. *Drosophila* transgenic lines were created carrying genes for human kinases, including GSK3β, PKA, CK1δ and DYRK1A. The kinase transgenes were inserted in different genomic positions at random and most of them carried a tag to detect their expression on western blot. The constructs encoding for human and rat kinases contained the same features as described above. The kinases were expressed in the *Drosophila* eye using the GMRGAL4 driver. Expression was induced throughout development at 25ºC and flies were collected between 0 to 5 days post-eclosion. The effect of co-expression of the above human kinases in *Drosophila* photoreceptor neurons were analysed by light microscopy and western blotting. Control flies expressing GMRGAL4 alone are characterised by an oval optic lobe and a regular arrangement of ommatidia (Figure 4.3A). When GSK3β, PKA, CK1δ and DYRK1A were individually expressed in the fly eye, no ommatidial disarrangement was observable compared to control (Figure 4.3B-E). Western blot analysis confirmed that expression of the kinases was driven in the eyes. Proteins were extracted from fly heads using standard protocols (Chapter 2). Human GSK3β and CK1δ were detected using anti-myc and anti-flag antibodies respectively. An antibody specific for the α-catalytic domain of PKA was used for the flies expressing human PKA. Finally, tagged rat DYRK1A was detected using an anti-flag antibody. Actin or tubulin expression were used to monitor protein loading. All the kinases were expressed and represented on the blots by a single band of expected molecular weight.

Transgenic lines for GSK3β, CK1δ and DYRK1A express considerable amount of kinases, in contrast the line for human PKA shows a weak kinase expression. Levels of expression varied as different insertion sites of the transgenes were used. Fly lines expressing the highest levels of human and rat kinases were selected to use in this study (data not shown). The above characterisation of *Drosophila* lines expressing human and rat kinases demonstrates that no alteration on the regular structure of ommatidia is caused by the transgene expression compared to control and that the fly lines have reproducible levels of transgene expression.

In conclusion, expression of human and rat kinases in *Drosophila* photoreceptor neurons does not affect any endogenous developmental pathway necessary for eye development.

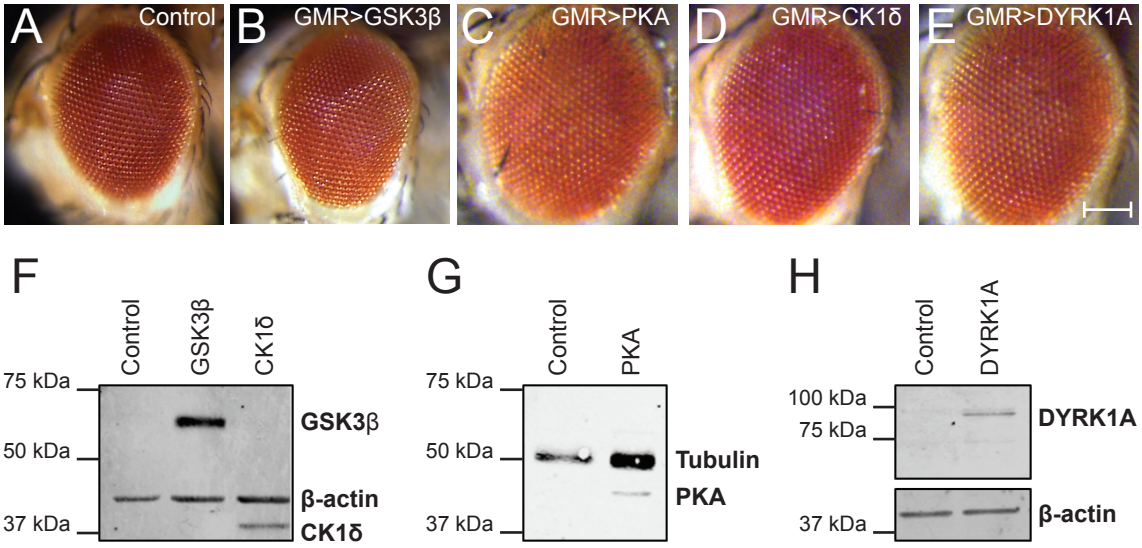

Figure 4.3 Expression of human kinases in the developing *Drosophila* eye does not alter the ommatidial architecture. (A-E) Light micrographs of eyes from 0-5 days old flies. (A) Control eyes carrying the eye specific GMRGAL4 driver show a regular array of ommatidia. (B-F) Drosophila transgenic lines expressing the human kinases (B) GSK3β, (C) PKA, (D) CK1δ and (E) the rat kinase DYRK1A does not cause ommatidial disarrangement compared to control. (F-H) Kinase expression in the Drosophila eye was detected by western blotting. β-actin or α-tubulin were used as loading controls (42 and 50 kDa respectively). Flies carrying the GMRGAL4 driver only were used as negative control. (F) Expression of human GSK3β and CK1δ were detected using anti-myc and anti-flag antibodies (60 kDa and 37 kDa respectively). (G) Human PKA expression was detected using a specific antibody against the α chain (40 kDa). (H) Rat DYRK1A expression was detected using an anti-flag antibody (95 kDa). Scale bar: 100 μm. Dorsal is top and anterior is left.

Many studies have focused on the interaction of human tau and human kinases *in vitro*, however there is no *in vivo* evidence of which human kinases are involved in tau hyperphosphorylation or whether specific phosphorylation sites mediate toxicity (Anderton et al, 2001; Cruz & Tsai, 2004; Feijoo et al, 2005; Hanger et al, 2009; Hanger et al, 2007; Mandelkow et al, 2004). This study aims to identify the human kinases producing the most pathogenic tau species *in vivo*. To examine whether the introduced kinases can increase the 2N4R tau-mediated toxicity, they were co-expressed together with tau and the effect on the eye phenotype characterised.

Human 2N4R tau and kinases (GSK3β, PKA, CK1δ and DYRK1A) were expressed using GMRGAL4 that drives the expression of the transgenes in the fly eye. Gene expression was induced throughout development at 25ºC and flies were collected between 0 to 5 days post-eclosion. The eye phenotypes were examined by light microscopy. Control flies express GMRGAL4 alone in order to monitor the background phenotype caused by the expression of the genetic driver. Control eyes are characterised by an oval optic lobe and a regular arrangement of ommatidia (Figure 4.4A). Expression of tau alone from line 2N4Rtau#68A causes a weak disarrangement of the ommatidial pattern compared to control (Figure 4.4B). The *Drosophila* lines expressing human and rat kinases were demonstrated to present an unaltered eye structure compare to control (Figure 4.3A-E). Human 2N4R tau was co-expressed with each individual kinase in order to see whether the eye degeneration would increase upon interaction of human tau and kinases. When human GSK3β is co-expressed with human 2N4R tau, the eye degeneration phenotype is enhanced compared to human tau alone. The disarrangement of ommatidia is increased in the anterior part of the eye and the characteristic oval shape of the eye is altered compared to control (Figure 4.4C). However, human PKA and human CK1δ co-expression with tau does not lead to an enhancement of eye degeneration. Unexpectedly, the level of degeneration caused by the interaction of 2N4R tau and PKA appears to be lower compared to tau alone and more similar to control (Figure 4.4D). Moreover, co-expression of 2N4R tau and CK1δ does not affect the basal human tau-mediated eye degeneration caused by line 2N4Rtau#68A (Figure 4.4E). Finally, rat DYRK1A co-expression causes a minor enhancement of human tau-mediated degeneration shown by increased ommatidial disarrangement in the anterior part of the eye (Figure 4.4F).

Surprisingly, not all the kinases increase tau toxicity in this model. Human GSK3β and rat DYRK1A produced an increased degeneration indicating increased tau toxicity in the fly eye. The kinase showing the greatest enhancement of human tau-mediated eye degeneration was GSK3β, while DYRK1A produced a very mild phenotypic enhancement. In contrast, PKA and CK1δ did not increase the eye degeneration compared to when human tau is expressed alone.

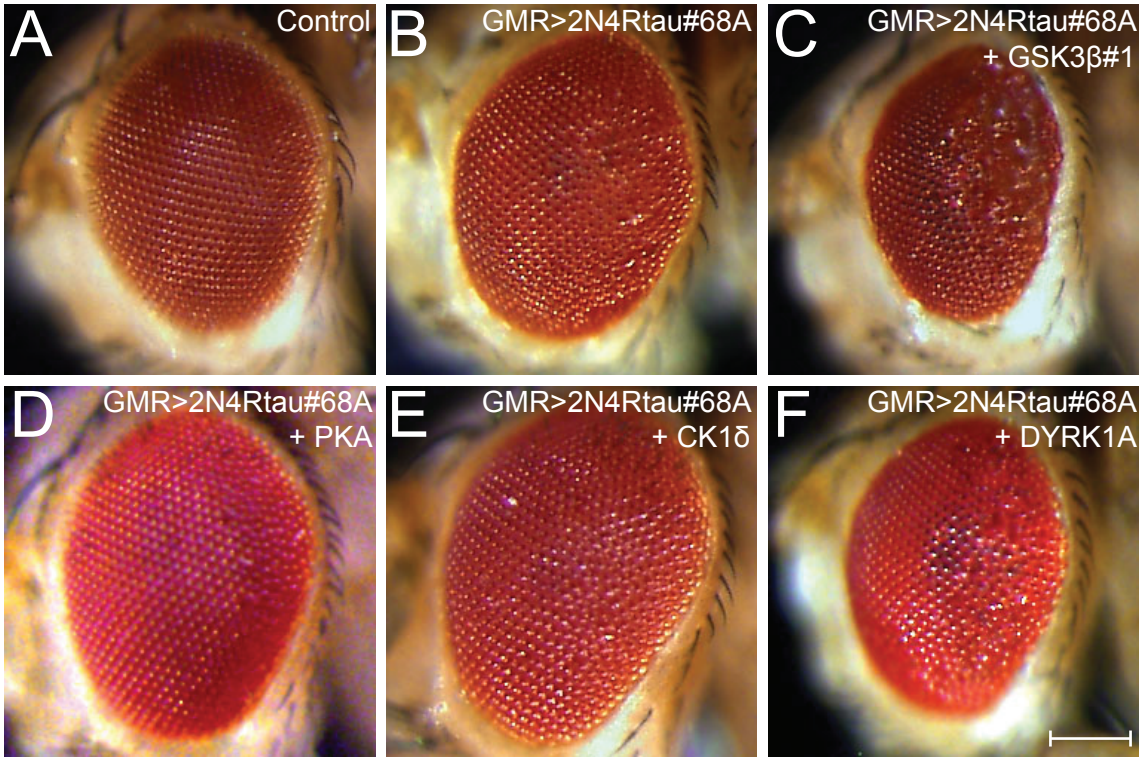

Figure 4.4 Human tau-mediated degeneration is enhanced by co-expression of human and rat kinases. Light micrographs of eyes from 0-5 days old flies. (A) Control eyes carrying the eye specific GMRGAL4 driver show a regular array of ommatidia. (B) Expression of human tau from 2N4Rtau#68A causes weak ommatidial disruption. (C) Co-expression of human 2N4R tau and human GSK3β enhances the tau-mediated degeneration in the eye. (D) No ommatidial disruption is shown when PKA is co-expressed with human tau. (E) Co-expression of human 2N4R tau and CK1δ causes a weak disruption of ommatidia. (F) Eye degeneration is slightly enhanced when DYRK1A is co-expressed together with human tau. Scale bar: 100 μm. Dorsal is top and anterior is left.

4.2.2 Human tau and GSK3β in the fly eye

The previous section revealed an enhancement of tau-mediated degeneration when GSK3β is also expressed alongside the human 2N4R tau. In order to confirm the human GSK3β enhancement, the expression of the kinase was modulated and the resulting phenotype observed. Several fly lines carrying the same human GSK3β transgene were generated using standard P-element insertion with the transgene inserted in different genomic positions.

Transgene expression was controlled by the eye specific GMRGAL4 driver and was induced throughout development at 25ºC. Flies were collected between 0 to 5 days post-eclosion and they were analysed by light microscopy and western blotting. Control flies express GMRGAL4 alone and exhibit an oval optic lobe and a regular arrangement of ommatidia (Figure 4.5A). All lines expressing human GSK3β alone (lines GSK3β#1-4) showed an eye phenotype similar to control revealing that the expression of the human kinase does not interfere with the fly eye development (Figure 4.5B-E). As shown before, line 2N4Rtau#68A is characterised by a weak disarrangement of the ommatidial pattern compared to control (Figure 4.5F). When line 2N4Rtau#68A was combined with the lines expressing human GSK3β, the tau-mediated eye degeneration was enhanced (Figure 4.5G-J). Interestingly, the level of enhancement of the degenerative phenotype was different in the four lines co-expressing human tau and GSK3β. The fly line producing the greatest level of degeneration was GSK3β#1, as shown in figure 4.4C. Mild enhancement was observed when human tau was expressed together with GSK3β#2 and GSK3β#3. Finally, expression of GSK3β#4 did not enhance the eye degeneration shown when human tau is expressed alone.

The transgenic lines for GSK3β carry the human kinase gene inserted in different genomic positions, therefore, its expression level is different in each line (as demonstrated in Chapter 3). The protein levels for GSK3β were tested in all transgenic lines by western blotting (Figure 4.5K). Human GSK3β was detected using an anti-myc antibody. Control flies show no GSK3β expression. Following expression using the GMRGAL4 driver, human GSK3β is detected in all transgenic lines (GSK3β#1-4) as single band at the expected molecular shift. Phosphorylation of GSK3β on S9 has been

used as marker to monitor its activity; when S9 is phosphorylated GSK3β is not active. In order to see whether human GSK3β was active when expressed in the *Drosophila* eye, the level of phosphorylation of S9 was tested by western blotting. A phospho-specific antibody for human GSK3β-pS9 detected a single band at the same molecular shift of total GSK3β. The blot revealed a very low level of phosphorylation of S9 compared to the amount of total GSK3β expressed, suggesting that the human kinase is not inactivated in fly cells (Figure 4.5K). Expression levels of GSK3β were quantified using actin to normalise for gel loading. To enable a better comparison between the different lines, the protein levels were expressed in percentage relative to the highest mean. Line GSK3β#1 expresses a significantly higher amount of human kinase compared to the other lines, while GSK3β#4 the lowest amount (Figure 4.5L).

In conclusion, the level of increased tau toxicity correlates to the amount of GSK3β expressed. This evidence shows that human 2N4R tau and GSK3β interacts together leading to a greater degeneration in the *Drosophila* eye. Moreover, human GSK3β is not inactivated by phosphorylation of S9 in a *Drosophila* background. Line GSK3β#1 was used in the following experiments because of its high level of human kinase expressed.

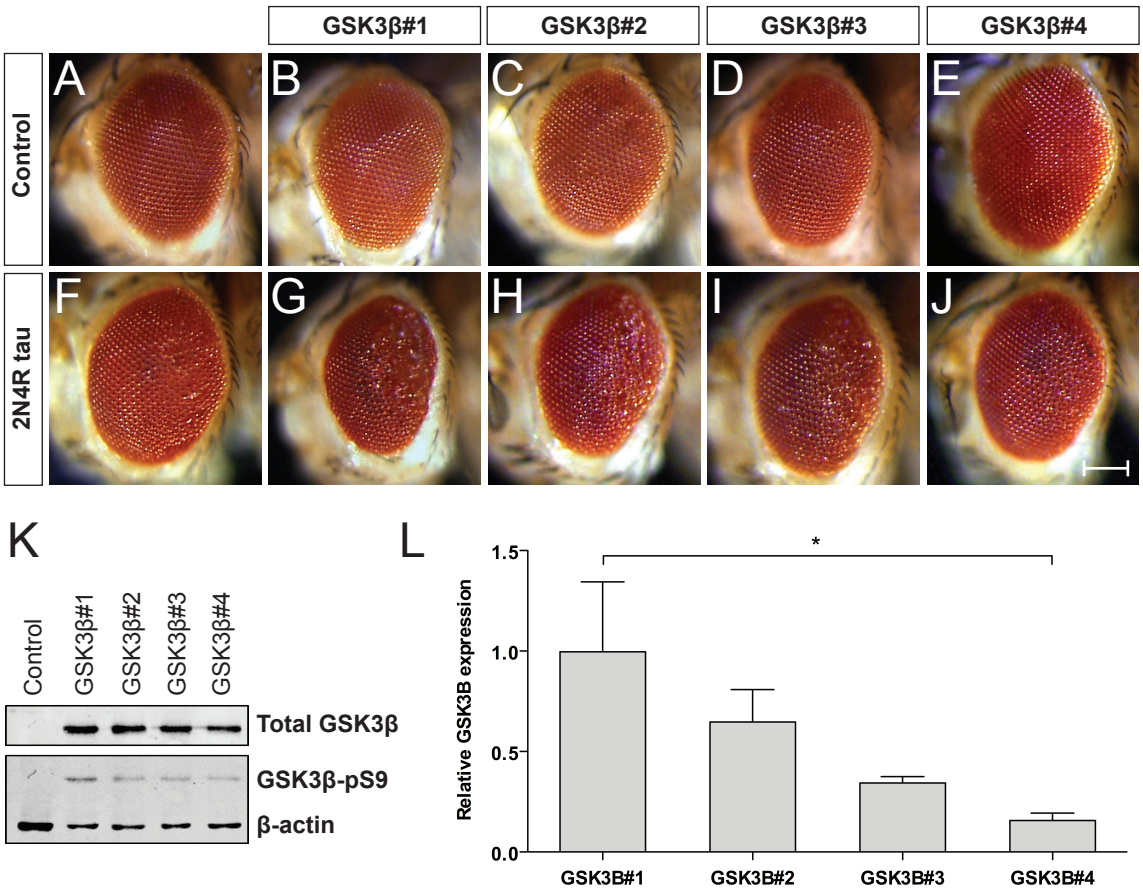

Figure 4.5 Human GSK3β enhances tau-mediated degeneration in the *Drosophila* eye proportionally to its expression level. Light micrographs of eyes from 0-5 days old flies. (A) Control eyes carrying the eye specific GMRGAL4 driver show a regular array of ommatidia. (B-E) Expression of human GSK3β from three different insertion sites in the Drosophila eye using GMRGAL4 causes no change in regular ommatidial arrangement. (F) Expression in the eye of the human tau transgene in the 2N4Rtau#68A transgenic line causes a weak ommatidial disruption. (G-J) Co-expression of human 2N4R tau and human GSK3β enhances the tau-mediated degenerative phenotype in the eye. (K) Human GSK3β and actin expression were detected by western blotting from head homogenates. flies carrying GMRGAL4 alone were analysed as negative control. Human GSK3β was detected as a single band (60 kDa) using an anti-myc antibody (total GSK3β) and a phospho-specific antibody for S9 (GSK3β-pS9) which reveals inactivation of the kinase. β-actin was used as loading control (42 kDa). (L) GSK3β protein levels for each transgenic line were normalised to actin. Values correspond to the mean and SEM of 3 replicates of independent experiments. To enable comparison between samples, the protein levels were expressed as relative numbers to the highest mean. Asterisks indicate statistically significant differences of protein expression. The levels of human GSK3β expression are proportional to the enhancement of eye degeneration caused when co-expressed with human tau. The levels of S9 phosphorylation on GSK3β are very low suggesting the kinase is not activated. Scale bar: 100 μm. Dorsal is top and anterior is left.

Co-expression of human GSK3β with human 2N4R tau causes an enhancement of tau toxicity in the *Drosophila* eye. I next examined the toxic modifications on tau caused by GSK3β expression. Alterations in the solubility properties of tau has been shown to be associated with increased toxicity in disease and one of the major factors contributing to this alteration is tau phosphorylation (Hirata-Fukae et al, 2009; Ishihara et al, 2001; Lewis et al, 2000; Zhukareva et al, 2002). In Chapter 3 of this study, the *Drosophila* lines over-expressing human 2N4R tau were shown to produce Sarcosyl-soluble and insoluble tau species.

I examined whether the levels of Sarcosyl-soluble and insoluble human tau change upon human GSK3β expression in the fly eye. Protein homogenate obtained from fly heads was resuspended in 1% Sarcosyl and insoluble tau separated by centrifuging at 100,000g (Chapter 2). Human 2N4R tau (line 2N4Rtau#68A) and human GSK3β (line GSK3β#1) were expressed throughout development at 25ºC with the eye-specific GMRGAL4 driver. Protein extracts were prepared from heads of flies collected 0 to 5 days post-eclosion. Sarcosyl-soluble and insoluble human tau was successfully detected on western blot and the expression levels were quantified using actin to normalise for protein loading (Figure 4.6). The quantification reveals that the levels of soluble and insoluble tau do not change when GSK3β is co-expressed compared to human tau alone. However, the levels of insoluble tau produced are significantly lower than the levels of soluble tau, as previously shown in figure 3.9. This result shows that the phosphorylation by *Drosophila* endogenous kinases does induce the formation of Sarcosyl-insoluble tau species, however these do not appear to be enriched after expression of human GSK3β. More importantly, here I show that tau-mediated degeneration induced by human GSK3β occurs without a shift in human tau solubility in flies. This does not exclude the possibility that GSK3β phosphorylates tau on key sites that increase its toxicity in the fly eye.

In section 4.2.1 I demonstrated that in S2 cells human GSK3β increases the phosphorylation of 2N4R tau at specific sites. Therefore, I examined whether the same sites are phosphorylated *in vivo* and whether these forms distribute equally in the soluble and insoluble fractions of tau. As for the experiment conducted in S2 cells, I initially planned to map the phosphorylated sites on human tau extracted from fly heads via mass spectrometry. Due to the impossibility of extracting enough protein to perform

the analysis, only a limited number of phosphorylation sites were examined using the same phospho-specific antibodies as for section 4.2.1: PHF1 (pS396 and pS404), AT8 (pS202 and pT205), AT270 (pT181) and AT100 (pS212 and pS214). The tau antibody supplied by Dako was used to detect the total level of human tau. Expression of human tau was successfully detected on western blot (Figure 4.7). Control flies carrying the GMRGAL4 driver only show no tau expression in the soluble or insoluble fractions. Following expression using GMRGAL4, human 2N4R tau was detected as single band in all samples at an apparent molecular weight of 65 kDa corresponding to recombinant 2N4R tau. The tau mobility does not change when GSK3β is co-expressed. Previous studies have shown that hyperphosphorylated tau is insoluble and characterised by a higher shift weight compared normal tau (Ishihara et al, 2001; Lewis et al, 2000; Zhukareva et al, 2002). This result confirms that the activity of only one kinase is not enough to generate hyperphosphorylated forms of human tau in the cell. Moreover, human tau is differently represented on western blot when extracted from S2 cells compared to fly eyes. In S2 cells transfected 2N4R tau is found in multiple bands suggesting a differential phosphorylation by endogenous *Drosophila* kinases (Figure 4.1), while, when expressed in fly eyes, it appears as a single band. A different set of endogenous kinases might be active in S2 cells compared to the eye possibly leading to a different pattern of phosphorylation of human tau.

The phosphorylation of human tau by GSK3β was examined on disease-relevant epitopes as follows. Phosphorylation at PHF1 epitopes is specifically detected in the soluble and insoluble tau fractions of flies over-expressing human 2N4R tau and GSK3β (Figure 4.7A), while the AT8 epitope is phosphorylated also when human tau is expressed alone in the eye but not in S2 cells (Figure 4.7B). Also AT270 detects phosphorylation of soluble and insoluble tau in all samples but it appears to be increased in flies co-expressing human tau and GSK3β (Figure 4.7C). In contrast, phosphorylation at AT100 epitopes is very low in the soluble tau fraction and is not present in the insoluble fraction (Figure 4.7D). The increase in phosphorylation in the soluble and insoluble tau fractions was measured when GSK3β was co-expressed together with tau in the fly eye. The phosphorylation levels for PHF1, AT8 and AT270 were quantified using total tau and actin to monitor gel loading (Figure 4.7E and F). In the soluble fraction of tau the levels of phosphorylation at PHF1 and AT270 epitopes are significantly increased upon human GSK3β expression. In particular, the PHF1

epitope is not phosphorylated in flies expressing human tau only, while it shows a basal level of phosphorylation in S2 cells transfected with human tau only (Figure 4.1B). A basal level of phosphorylation by endogenous kinases contributes to phosphorylation at the AT270 epitope in the fly eyes as seen previously in S2 cells (Figure 4.1D). In contrast, the AT8 epitope shows a non-significant increase in phosphorylation when GSK3β is co-expressed, differently from the result obtained in S2 cells where phosphorylation of this epitope was specific for GSK3β (Figure 4.1C). The insoluble fraction of tau appears to be characterised by the same phosphorylation pattern. PHF1 and AT270 epitopes are characterised by a significant increase in phosphorylation when GSK3β is expressed, while phosphorylation at the AT8 epitope does not show a significant change.

Thus, the proportions of Sarcosyl-soluble and insoluble human tau do not change upon GSK3β expression. The increased tau toxicity caused by human GSK3β in the fly eye is not due to a shift in solubility of tau. Moreover, human GSK3β can phosphorylate 2N4R tau in *Drosophila*. In particular, phosphorylation at PHF1 and AT270 epitopes are significantly increased when GSK3β is expressed in the eye differs to that seen in S2 cells. In conclusion, human tau is unlikely to undergo hyperphosphorylation via GSK3β in flies since its solubility is unaltered. However, more specific phosphorylation events might be responsible for the increased level of toxicity seen in the fly eye when human GSK3β is co-expressed with human 2N4R tau.

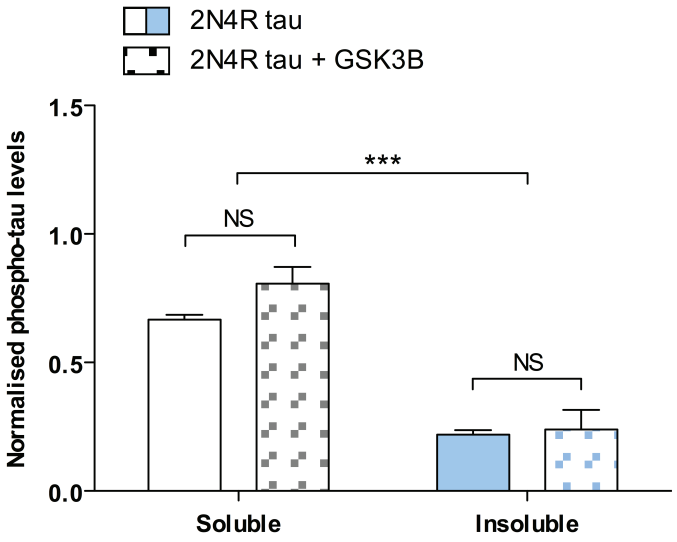

Figure 4.6 The amount of soluble and insoluble tau does not vary upon human GSK3β expression in *Drosophila*. Human tau and human GSK3β were expressed in the fly eye using the GMRGAL4 driver. Human tau and actin were detected on western blot. Protein levels were normalised to actin. Values correspond to the mean and SEM of 3 replicates of independent experiments. Asterisks indicate statistically significant differences of protein expression. The histogram shows the amount of Sarcosyl-soluble and insoluble human tau expressed with or without GSK3β co-expression. The levels of soluble and insoluble human tau do not vary depending on GSK3β co-expression.

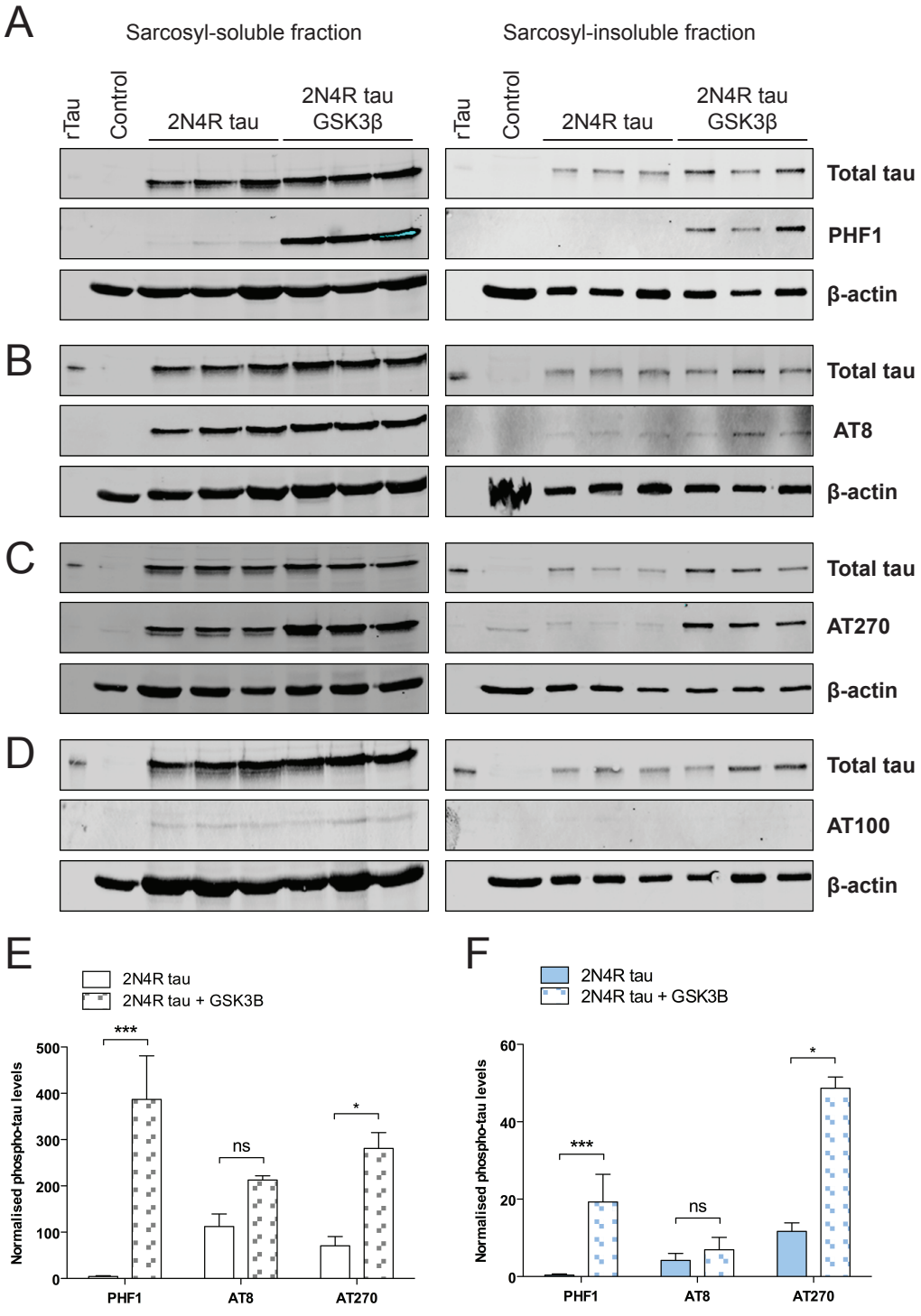
A
Sarcosyl-soluble fraction
Sarcosyl-insoluble fraction
rTau
Control
2N4R tau
2N4R tau GSK3β
Total tau
PHF1
β-actin
B
Total tau
AT8
β-actin
C
Total tau
AT270
β-actin
D
Total tau
AT100
β-actin
E
2N4R tau
2N4R tau + GSK3B
Normalised phospho-tau levels
500
400
300
200
100
0
***
ns
*
PHF1
AT8
AT270
F
2N4R tau
2N4R tau + GSK3B
Normalised phospho-tau levels
60
40
20
0
***
ns
*
PHF1
AT8
AT270

Figure 4.7. Human GSK3β increase the phosphorylation level of human tau in *Drosophila*. Human tau and human GSK3β were expressed in the fly eye using the GMRGAL4 driver. Human tau and actin expression were detected by western blotting. Recombinant human 2N4R tau (rTau) was loaded as positive control and flies carrying GMRGAL4 alone were analysed as negative control. Sarcosyl-soluble and insoluble tau fractions were analysed. Human tau was detected as a single band (60 kDa) using the phospho-independent antibody supplied by Dako. β-actin was used as loading control (42 kDa). Phosphorylated tau was also detected using (A) PHF1 (B) AT8 (C) AT270 (D) AT100. (E-F) Levels of phospho-tau in the Sarcosyl-soluble and insoluble fractions. Protein levels were normalised to actin. Values correspond to the mean and SEM of 3 replicates of independent experiments. Asterisks indicate statistically significant differences of protein expression. Co-expression of GSK3β together with human tau increases the phosphorylation levels of PHF1 and AT270 epitopes in both tau fractions. The AT8 epitope remains unchanged, while no phosphorylation is seen at the AT100 epitope.

Recent studies have suggested that specific kinases may prime tau for phosphorylation by other kinases *in vitro*. For example, phosphorylation by GSK3β on tau might be primed by phosphorylation via CK1δ or DYRK1A (Li et al, 2006; Woods et al, 2001). I tested whether multiple kinases have an additive or synergistic effect on human tau toxicity. The *Drosophila* model created in this study was used to assess whether GSK3β-mediated eye degeneration could be enhanced by co-expression of CK1δ or DYRK1A.

Human 2N4R tau and pairs of kinases were expressed in the fly eye using the GMRGAL4 driver. Gene expression was induced throughout development at 25ºC and flies were collected between 0 to 5 days post-eclosion. The eye phenotypes were examined by light microscopy. Control flies express GMRGAL4 alone in order to monitor the background phenotype caused by the expression of the genetic driver. Control eyes are characterised by an oval optic lobe and a regular arrangement of ommatidia (Figure 4.8A). As shown before, line 2N4Rtau#68A is characterised by a weak disarrangement of the ommatidial pattern compared to control (Figure 4.8B). When human GSK3β is co-expressed with human 2N4R tau, the eye degeneration phenotype is enhanced compared to human tau alone (Figure 4.8C). Moreover, human CK1δ co-expression with tau does not lead to an enhancement of eye degeneration, while rat DYRK1A co-expression causes a minor enhancement of human tau-mediated degeneration (Figure 4.8E). When human 2N4R tau was co-expressed with GSK3β and CK1δ, the eye degeneration phenotype was increased compared to human tau and CK1δ expression. Moreover, the intensity of the degeneration was also mildly enhanced compared to human tau and GSK3β expression. The disarrangement of the ommatidia spread to the whole surface of the eye, while it is concentrated to the anterior part of the eye upon tau and GSK3β expression. In contrast, the eye phenotype caused by expression of human tau, GSK3β and DYRK1A was very similar to the phenotype caused by human tau and DYRK1A alone but milder compared to the one cause by GSK3β alone.

In conclusion, GSK3β and CK1δ or DYRK1A do not have a synergistic effect in enhancing human tau-mediated toxicity. In fact, the eye phenotype resulting from the co-expression of GSK3β and CK1δ or DYRK1A together with human tau did not show

a very significant exacerbation of tau toxicity that would be expected if those kinases would have a priming effect on one another.

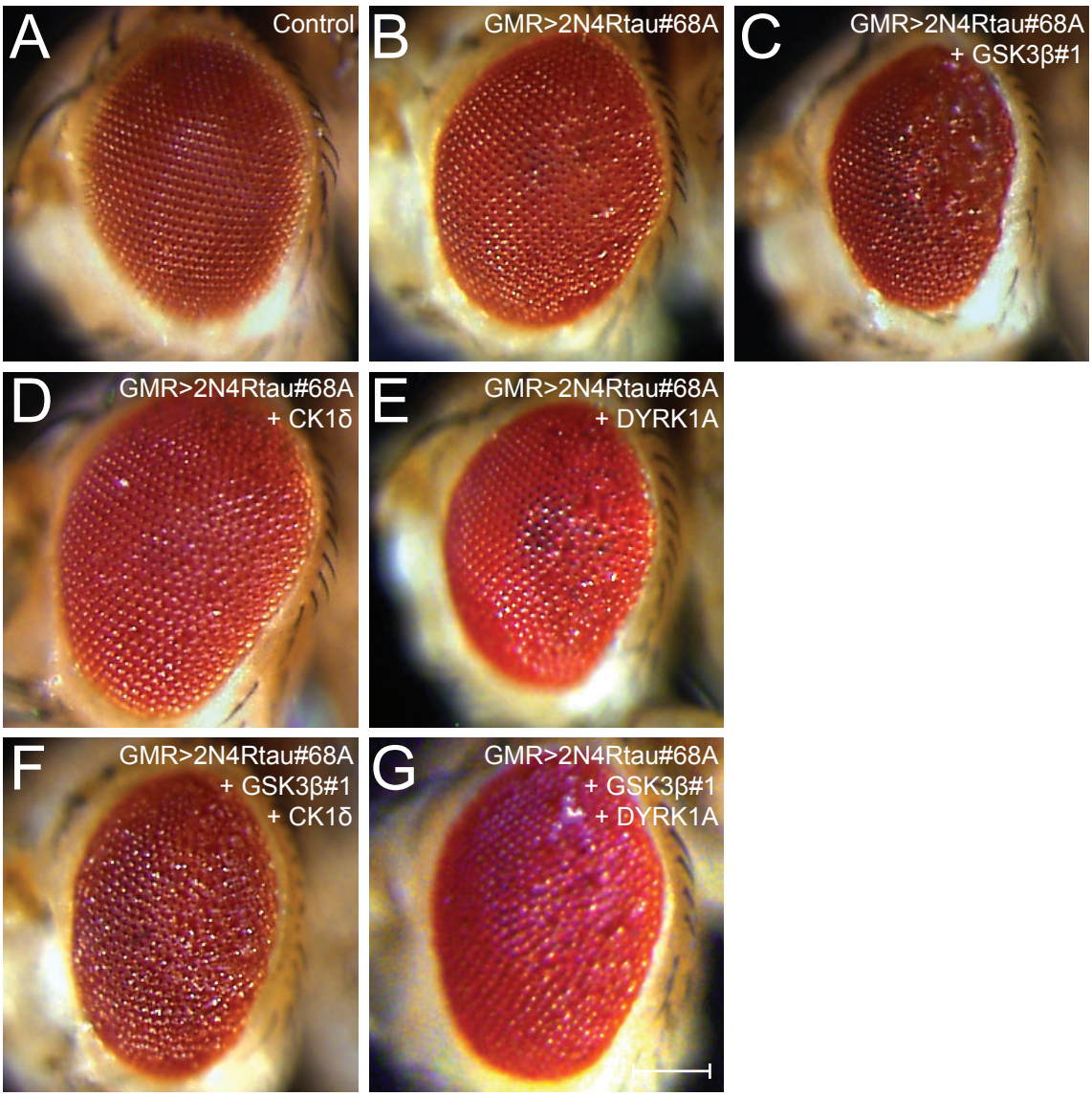

Figure 4.8 Co-expression of additional kinases does not enhance the human tau-mediated degeneration produced by GSK3β. Light micrographs of eyes from 0-5 days old flies. (A) Control eyes carrying the eye specific GMRGAL4 driver show a regular array of ommatidia. (B) Expression of human 2N4R tau causes weak ommatidial disruption. (C) Co-expression of human 2N4R tau and human GSK3β enhances the tau-mediated degeneration in the eye. (D) Co-expression of human 2N4R tau and CK1 δ causes a weak disruption of ommatidia. (E) Eye degeneration is slightly enhanced when DYRK1A is co-expressed together with human tau. The tau-mediated degeneration is not further enhanced by the co-expression of (F) CK1δ or (G) DYRK1A together with 2N4R tau and GSK3β. Scale bar: 100 μm. Dorsal is top and anterior is left.

4.2.3 Effect of tau point mutations on GSK3β-mediated toxicity

To investigate the role of individual phosphorylated residues in the production of toxic tau, several constructs for mutated tau were generated. As seen above, human GSK3β increased tau-mediated toxicity in flies and potential phosphorylation sites involved in conferring toxicity were identified on human tau via western blotting. Phosphorylation of S396 and S404, corresponding to the PHF1 epitope, was confirmed to be increased by GSK3β in both S2 cells and fly eyes. However, the AT8 epitope at pS202 and pT205 showed opposite results in S2 cells compared to the *in vivo* experiments. Thus, it is still unclear whether these sites are important in increasing tau toxicity via GSK3β. Moreover, T181 phosphorylation recognised by AT270 has been shown to occur by GSK3β and also endogenous kinases in both S2 cells and fly eye. Finally, previous studies have identified T212 on tau as one of the major phosphorylation site targeted by the novel kinase DYRK1A. Furthermore, phosphorylation of this site might influence phosphorylation by GSK3β on human tau (Woods et al, 2001). In light of my previous experiments looking at the effect of concurrent phosphorylation by GSK3β and DYRK1A on human tau, T212 appears to be an important site to further examine.

Four tau constructs were created carrying point mutations substituting serine or threonine sites with alanines in order to inhibit phosphorylation of these sites. The mutations generated by *in vitro* mutagenesis are S202A, T205A, T212A and S404A. Another construct was created carrying all four of those mutations, including 202, 205, 212 and 404 (4xA). Unfortunately, due to time constraints, I was not able to examine the effect of the mutation T181A. In section 4.2.1 it was shown that in *Drosophila* S2 cells the interaction of human tau with human GSK3β causes a shift in mobility of tau probably due to phosphorylation. In order to see whether the mutated constructs of human tau were modified differently by human GSK3β, S2 cells were transfected with wild type and mutated 2N4R tau with or without GSK3β. As previous experiments in S2 cells, expression of human 2N4R tau was controlled by an inducible UAS-GAL4 system and expression of human GSK3β by an actin promoter. GSK3β carried a 3' end tag made by a myc epitope. Protein expression occurred at 25ºC. Expression of wild type and mutated human 2N4R tau was induced for five hours while human GSK3β was constitutively expressed. Expression of human tau and GSK3β was successfully

detected by western blotting (Figure 4.9A). The phospho-independent tau antibody recognises the total level of tau expressed. Human GSK3β was detected using an anti-myc antibody and it migrated at the expected molecular weight (60 kDa). Actin expression was used to monitor protein loading. Control cells carrying an empty vector show only actin expression. Due to different levels of basal phosphorylation by endogenous kinases in S2 cells, wild type human tau is detected on the blot as multiple bands ranging from 60 kDa to 65 kDa. Moreover, human tau carrying S202A, T205A and T212A also exhibit a multiple band pattern on western blot similar to wild type tau. In contrast, human tau with the S404A mutation and 4xA tau were detected on the blot as single bands of low molecular weight (60 kDa). When wild type and mutated 2N4R tau were co-transfected together with human GSK3β, a molecular shift was observed for wild type 2N4R tau, tau S202A, tau T205A and tau T212A as revealed using the a phospho-independent tau antibody (Figure 4.9A). One additional species of human tau S404A was produced at higher molecular weight upon GSK3β expression. In contrast, 4xA tau did not show any molecular shift when GSK3β is expressed.

In order to monitor the phosphorylation level of wild type and mutated 2N4R tau, the same samples were analysed with phospho-specific antibodies for human tau including PHF1 (pS396 and pS404), AT8 (pS202 and pT205), AT270 (pT181), AT100 (pS212 and pS214) (Figure 4.9B-E). Control cells carrying an empty vector show no phospho-tau expression. Phosphorylation at the PHF1 epitope only occurred in the samples where GSK3β was co-expressed (Figure 4.9B). The antibody did not recognise human tau mutated with S404A since phosphorylation is now inhibited at that site. Moreover, the AT8 antibody detected a very mild phosphorylation of wild type, S212A and S404A tau upon GSK3β expression. Human tau with S202A and T205A mutations were not detected indicating that phosphorylation at these sites is inhibited. However, the AT270 antibody recognises a baseline phosphorylation level on human tau by endogenous kinases. Its level of detection is increased when GSK3β is co-expressed, except for 4xA tau (Figure 4.1D). The AT100 antibody shows no detection as seen in previous experiments using in S2 cells (Figure 4.1E).

These results, particularly figure 4.9A, suggest that mutating S404 on 2N4R tau inhibits the basal level of phosphorylation by endogenous kinases and human GSK3β in fly cells. The individual mutations S202A, T205A and T212A do not affect

phosphorylation by GSK3β on other sites. However, when mutated all together S202, T295, T212 and S404 appear to affect phosphorylation of the AT270 epitope. All together these results lead to the conclusion that phosphorylation of S404 might play a key role in favouring the phosphorylation of other sites on human tau by endogenous *Drosophila* kinases leading to hyperphosphorylation.

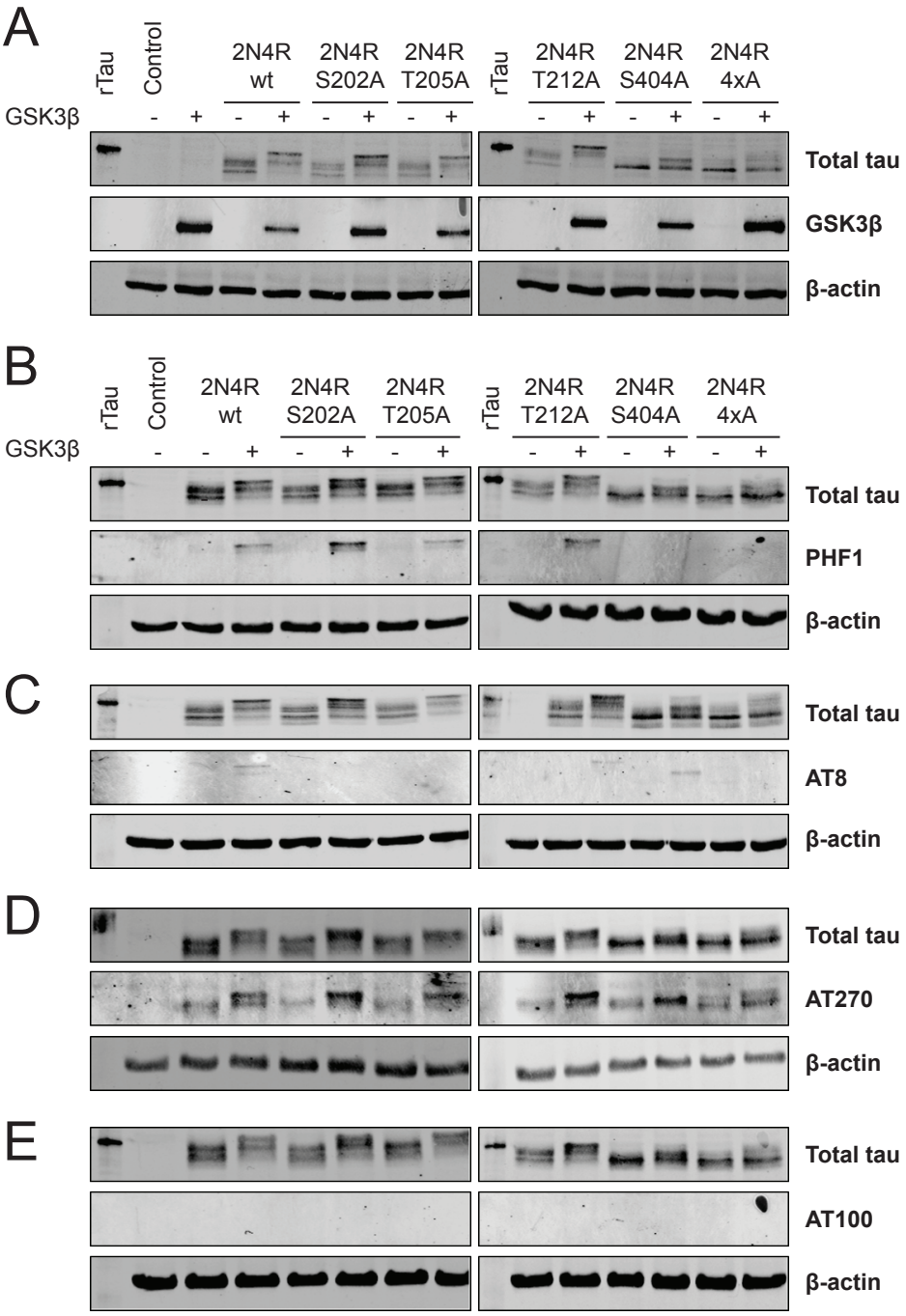

Figure 4.9 Point mutations on human tau reveals a key role for S404 in the tau phosphorylation generated by GSK3β in Drosophila S2 cells. Constructs bearing the single mutations S202A, T205A, T212A, and S404A or all four mutations (4xA) were co-transfected with GSK3β into S2 cells. Recombinant human 2N4R tau (rTau) was loaded as positive control for tau and S2 cells transfected with an empty vector were used as negative control. Total tau was detected as multiple bands (60-70 kDa) using the phospho-independent antibody Dako. β-actin was used as loading control (42 kDa). (A) GSK3β was detected using an anti-myc antibody (60 kDa). (B-E) Human tau was detected using phospho-specific antibodies (B) PHF1 for pS396 and pS404, (C) AT8 for pS202 and pT205, (D) AT270 for pT181 and (E) AT100 for pT212 and pS214. Human tau S404A and 4xA do not show a significant mobility shift when co-transfected with GSK3β compared to wild type tau.

In order to assess whether the point mutations previously generated affect the human tau-mediated degeneration in the fly eye, transgenic *Drosophila* lines were created carrying the mutated forms of human tau. Using the phiC31-mediated transgenesis, five constructs carrying mutated forms of human tau were inserted in the same genomic locus used by line 2N4Rtau#68A resulting in fly lines with the same genetic background and expression level of tau. Therefore, the tau-mediated toxicity in the fly eye is affected only by the mutation on human tau avoiding any positional effect of the transgene. The aim of this experiment is to assess whether the point mutations in human tau influence its interaction with the kinases thought to play a role in hyperphosphorylation of tau during disease.

The transgenes were expressed using GMRGAL4 that drives the expression of the transgenes in the fly eye. Gene expression was induced throughout development at 25ºC and flies were collected between 0 to 5 days post-eclosion. The eye phenotypes were examined by light microscopy. As shown before, line 2N4Rtau#68A is characterised by a weak disarrangement of the ommatidial pattern compared to control (Figure 4.10A1). When mutated forms of human tau are expressed in the fly eye, the phenotype exhibits comparable characteristics to those of 2N4Rtau#68A (Figure 4.10B1, C1, D1 and E1). The individual point mutations S202A, T205A, T212A and S404A on 2N4R tau cause a mild alteration of the eye architecture. The transgenic lines over-expressing human GSK3β, PKA, CK1δ and rat DYRK1A were crossed to the tau lines carrying the point mutations. When human GSK3β is co-expressed with human 2N4R tau, the eye degeneration phenotype is enhanced compared to human tau alone (Figure 4.10A2). Also, the eye degeneration of flies expressing mutated tau and human GSK3β is enhanced. The degree of enhancement of eye degeneration in flies carrying the S202A, T205A and T212A mutations is comparable to the enhancement seen in the line expressing wild type tau. The disarrangement of ommatidia is increased in the anterior part of the eye and the characteristic oval shape of the eye is altered compared to control (Figure 4.10 B2, C2 and D2). Surprisingly, the mutation S404A on tau appears to show a greater enhancement when human GSK3β is co-expressed. In fact, the ommatidial disarrangement extends to the central part of the eye and a significant depigmentation is observed (Figure 4.10E2). As in previous experiments human PKA co-expression with 2N4R tau does not lead to an enhancement of eye degeneration (Figure 4.10A3). Moreover, the tau lines carrying the individual mutations S202A, T205A and T212A

show no enhancement in eye phenotype when PKA is co-expressed (Figure 4.10B3, C3, D3). S404 is not a known site targeted by PKA phosphorylation, therefore it was not analysed (Figure 4.10E3). Subsequently, co-expression of wild type, T212A and S404A tau together with CK1δ does not affect the basal human tau-mediated eye degeneration caused by line 2N4Rtau#68A (Figure 4.10, panel A4, D4 and E4). Human CK1δ does not phosphorylate S202A and T205A, therefore these sites were not analysed (Figure 4.10B4 and C4). It has been shown before in this study that rat DYRK1A co-expression with 2N4R tau causes a minor enhancement of human tau-mediated degeneration (Figure 4.10A5). None of the individual mutations analysed alters the tau interaction with DYRK1A. The eye phenotypes are characterised by a minor enhancement of human tau-mediated degeneration comparable to that seen for line 2N4Rtau#68A (Figure 4.10 B5, C5, D5 and E5). The point mutations in 2N4R tau were also tested to alter the possible priming interaction of GSK3β by CK1δ or DYRK1A. Mutation on T212A appears not to change the enhancement of tau-mediated degeneration in flies expressing GSK3β and CK1δ in a 2N4Rtau#68A background (Figure 4.10A6 and D6). However, S404A tau exhibit a stronger enhancement in eye phenotype compared to wild type tau (Figure 4.10E6). Moreover, when wild type and mutated tau are expressed together with GSK3β and DYRK1A, the eye degeneration was similar to the phenotype caused in the wild type tau background, with exception for mutation S404A that shows a greater enhancement compared to the others (Figure 4.10, column 7).

These results show that single point mutations in 2N4R tau such as S202A, T205A and T212A do not affect the ability of human GSK3β, PKA, CK1δ and rat DYRK1A to generate toxic forms of human tau in *Drosophila*. Inhibiting phosphorylation of those sites does not change the eye degeneration enhancement caused by the kinases studied in this chapter. However, the mutation S404A appears to exacerbate the enhancement of phenotype generated by co-expression of human GSK3β and wild type 2N4R tau. The eye degeneration of flies expressing S404A tau and GSK3β is enhanced compared to the one shown by wild type tau and the same human kinase.

The use of single point mutations in tau did not alter the interaction between tau and endogenous kinases in flies, as previously shown by Steinhilb et al (2007a). Therefore, the effect of the four point mutations together on human tau was tested. Transgenic flies were generated expressing the human 2N4R tau carrying the following mutations:

S202A, T205A, T212A and S404A (4xA). The ability of the mutations to modulate tau toxicity via the expression of human kinases was also analysed. Transgene expression was controlled by the eye-specific GMRGAL4 driver at 25ºC. Flies were collected between 0 to 5 days post-eclosion. When tau 4xA is expressed in the fly eye alone, the phenotype is comparable to line 2N4Rtau#68A expressed alone. The eye is characterised by weak eye degeneration in the anterior part of the eye (Figure 4.11A1 and B1). Expression of tau 4xA together with human kinases showed a variation in degeneration phenotype only with GSK3β (Figure 4.11B1-7). The phenotype caused by human GSK3β in flies expressing human tau 4xA shows an increased level of degeneration compared to the one caused by line 2N4Rtau#68A (Figure 4.11B2). However, when human PKA, CK1δ and rat DYRK1A are expressed with tau 4xA the phenotype is identical to that seen when they are co-expressed with line 2N4Rtau#68A (Figure 4.11B3-5). When testing the combined action of CK1δ and DYRK1A with GSK3β, the degenerative phenotype generated using tau 4xA is enhanced over that caused by wild type tau (Figure 4.11B6 and B7). However, these enhancements are similar to that seen for tau 4xA and GSK3β alone suggesting that toxicity does not increase when other kinases are co-expressed with GSK3β. Interestingly, the degenerative enhancements obtained from the co-expression of human tau 4xA and human kinases are equivalent to those seen using human tau carrying the S404A mutation. Thus, this result leads to the conclusion that the enhancement of tau toxicity is only due to inhibiting phosphorylation or mutation of S404.

GSK3β
PKA
CK1δ
DYRK1A
GSK3β + CK1δ
GSK3β + DYRK1A
2N4Rtau#68A
S202A
T205A
T212A
S404A
A1
A2
A3
A4
A5
A6
A7
B1
B2
B3
B4
B5
B6
B7
C1
C2
C3
C4
C5
C6
C7
D1
D2
D3
D4
D5
D6
D7
E1
E2
E3
E4
E5
E6
E7

Figure 4.10. Effect of single tau point mutations on the degeneration caused by co-expression of human kinases. Light micrographs of eyes from 0-5 days old flies. All transgenes were expressed in the fly eye using the GMRGAL4 driver. (A1,B1,C1,D1 and E1) Expression of wild type and mutated 2N4R tau cause weak ommatidial disruption. (B2-D7) No alleviation of degeneration was observed when the single point mutations S202A, T205A and T212A were separately introduced on tau compared to wild type tau (A2-A7). (E2-E7) The single point mutation S404A increases the eye degeneration phenotype caused by GSK3β. Grey panels are located where the muatated site is not targeted by the kinase. Scale bar: 100 μm. Dorsal is top and anterior is left.

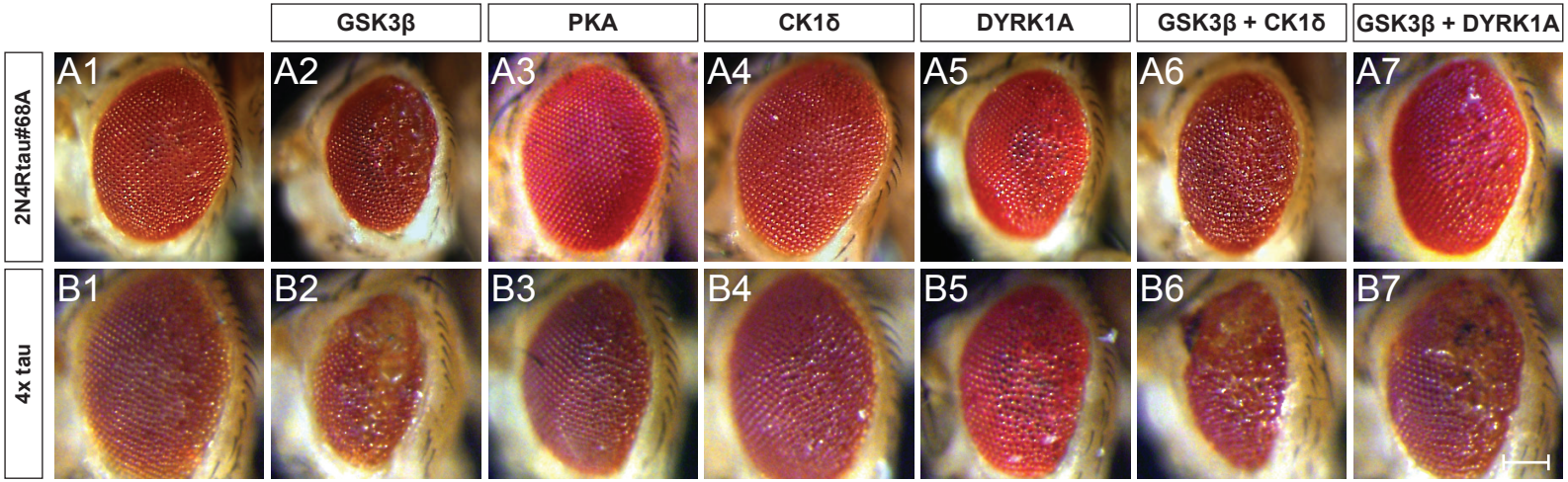

Figure 4.11 Multiple point mutations on human tau increase the eye degeneration generated by GSK3β. Light micrographs of eyes from 0-5 days old flies. All transgenes were expressed in the fly eye using the GMRGAL4 driver. (A1 and B1) Expression of wild type 2N4R tau or mutated tau carrying four point mutations (S202A, T205A, T212A and S404A) causes weak ommatidial disruption. (B2-B7) When tau 4xA is expressed together with individual kinases the degeneration is greater than that seen when wild type tau is expressed with the kinase (A2-A7). Scale bar: 100 μm. Dorsal is top and anterior is left.

4.3 Discussion

Neurofibrillary tangles are one of the main pathological features that contribute to the neurodegenerative process in AD. They are composed of hyperphosphorylated tau protein and the major kinases involved in the abnormal phosphorylation *in vivo* are unknown. Currently therapeutic strategies for AD are based upon preventing tau hyperphosphorylation by inhibiting the pathological kinases. *Drosophila* is now a well-established model for tauopathies in order to investigate tau hyperphosphorylation (Bonini & Fortini, 2003; Lau et al, 2002; Lewis et al, 2000; Muqit & Feany, 2002; Wittmann et al, 2001).

The aim of this chapter was to investigate the contribution made by individual human kinases to the degenerative phenotypes produced by human tau using the *Drosophila* eye as a measure to toxicity. The human kinases studied were selected from those that potentially contribute to tau pathology in humans, including GSK3β, PKA, CK1δ and DYRK1A. Fly lines over-expressing human tau and these kinases were characterised to assess any enhancement of tau-mediated eye degeneration. The kinase conferring increased toxicity was further analysed to examine the phosphorylation state of human tau. Finally, several tau point mutations on pathological S/T sites were tested for their ability to alter tau toxicity induced by the kinases.

4.3.1 Human tau and kinases co-expression in the *Drosophila* eye

Identifying the contribution of individual kinases to generate tau toxicity and their activity on the predicted sites *in vivo* is of crucial importance to develop new therapeutic strategies and identify biomarkers. Meaningful fly models of tauopathy have been created over-expressing human tau in the *Drosophila* photoreceptor neurons together with endogenous kinases (Bonini & Fortini, 2003; Jackson et al, 2002; Muqit & Feany, 2002; Wittmann et al, 2001). No evidence has been produced on the interaction of human tau and human kinases in *Drosophila* before.

In this study *Drosophila* transgenic lines over-expressing kinases including human GSK3β, PKA, CK1δ and rat DYRK1A were generated. When the human and rat kinases were over-expressed in the *Drosophila* eye, no toxicity was observed suggesting that those transgenes do not interact with any endogenous pathway in the developing eye. To confirm that tau toxicity can be enhanced in the tauopathy model created in this study, the level of the *Drosophila* orthologue of GSK3β, shaggy, were increased in the fly eye as previously done in other studies (Chatterjee et al, 2009; Jackson et al, 2002; Nishimura et al, 2004). As a result, the degeneration caused by human tau in the fly eye could be enhanced following hyperphosphorylation by an endogenous kinase. More importantly, the level of eye degeneration achieved overexpressing an endogenous kinase is not maximal, suggesting that the phosphorylation of tau by fly kinases does not saturate all sites available. Thus, it could be enhanced further by the introduction of other kinases. The ability of individual human kinases to increase tau toxicity was tested. The kinases PKA, CK1δ and DYRK1A did not show any considerable enhancement of eye phenotype. However, the only kinase able to enhance tau toxicity in the fly eye was human GSK3β. Co-expression of 2N4R tau with human GSK3β showed a considerable enhancement of eye degeneration. This result is perhaps not surprising considering that GSK3β is one of the best known kinases to play a crucial role in tau hyperphosphorylation during disease in humans (Hanger et al, 1992; Lovestone et al, 1994a; Lucas et al, 2001). In summary, GSK3β was the only human kinase conferring an increased toxicity to human tau in the *Drosophila* model of tauopathy created in this study. Therefore, further studies investigated any change in the biochemistry of human tau when GSK3β is co-expressed.

4.3.2 Phosphorylation and solubility of tau by GSK3β in *Drosophila*

Tau hyperphosphorylation and insoluble aggregates are two cellular features of tauopathies. It has already been demonstrated that expression of human tau in the *Drosophila* eye is toxic and causes neuronal degeneration (Chatterjee et al, 2009; Jackson et al, 2002; Steinhilb et al, 2007a; Steinhilb et al, 2007b; Wittmann et al, 2001). Moreover, it has been shown that kinase activity is important to generate tau toxicity since it causes hyperphosphorylation and alters tau solubility properties in mammals

and fly models (Chau et al, 2006; Hirata-Fukae et al, 2009; Jackson et al, 2002; Shulman & Feany, 2003).

Wittmann et al (2001) have shown that neurodegeneration occurs in flies expressing human tau without the formation of neurofibrillary tangles characteristic of human pathologies. However, tau aggregates have been found in the retina of transgenic *Drosophila* over-expressing human tau, suggesting the possible presence of pre-tangle tau forms. Moreover, human tau hyperphosphorylation by fly kinases has been shown to correlate with an alteration of tau protein solubility (Chau et al, 2006). In my model of tauopathy, human tau is predominantly found in a soluble state, while insoluble tau is produced in smaller amounts. The alteration in tau solubility is probably due to phosphorylation by endogenous kinases on human tau (Chatterjee et al, 2009; Chau et al, 2006). Moreover, in my thesis, changes in the proportion of soluble to insoluble human tau were measured upon co-expression of human GSK3β. Following western blot analysis, the levels of soluble and insoluble tau did not change when GSK3β was co-expressed compared to human tau expressed alone. As expected, the additional activity of only one kinase on tau does not alter its solubility confirming that the aberrant phosphorylation events leading to the formation of insoluble and aggregated tau forms in pathology are driven by the activity of several kinases. However, despite the absence of an alteration of tau solubility, human GSK3β increased the tau-mediated toxicity in the fly eye. Thus, this result suggests that a change in tau solubility is not needed to induce a greater level of tau toxicity in flies. It is possible that GSK3β phosphorylates tau on specific sites that increase tau toxicity without altering its solubility.

In *Drosophila* tauopathy models, human tau has already been demonstrated to be phosphorylated by endogenous kinases. The role of phosphorylation at serine-proline/threonine-proline (SP/TP) sites on human tau by *Drosophila* kinases has been proved to play a critical role in tau neurotoxicity in flies, but no-single phosphorylation residue seems to play a dominant role in controlling tau toxicity. Therefore, it could be likely that SP/TP sites cooperate to mediate neurodegeneration in the *Drosophila* eye when phosphorylated by endogenous kinases (Steinhilb et al, 2007a; Steinhilb et al, 2007b). In this thesis, human GSK3β is found to enhance tau toxicity beyond the level achieved by endogenous kinases. In this case there might be a possibility of identifying

single phosphorylated sites targeted by human GSK3β and contributing to the increase in toxicity driven by endogenous kinases. In this chapter, the phosphorylation of human tau on several residues was examined in the transgenic fly line expressing human tau and human GSK3β. I initially aimed to map the whole spectrum of phosphorylation sites on human tau via mass spectrometry. Unfortunately, due to technical reasons, I was not able to purify enough protein to perform the analysis. One of the reasons for this is that the construct with human tau did not carry any tag, which could have facilitated the protein extraction and purification. Therefore, the phosphorylated sites on human tau were mapped by western blotting using phospho-specific antibodies. The choice of the epitopes to examine was driven by the pathological relevance and availability of the antibodies. The antibodies PHF1 (pS396 and pS404), AT8 (pS202 and pT205) and AT270 (pT181) are widely used in the field of tauopathy research because phosphorylation of their epitopes is increased in human patients (Hasegawa et al, 1992; Lovestone & Reynolds, 1997). Phosphorylation of human tau by GSK3β was mapped *in vitro* and *in vivo Drosophila* systems. Interestingly, the phosphorylation pattern of human tau was different in S2 cells compared to the fly eye. This tissue-dependent phosphorylation is probably due to the activation of different sets of endogenous kinases in different fly cell types. *In vitro* in S2 cells, tau phosphorylation of the PHF1, AT8 and AT270 epitopes is increased upon GSK3β expression. However, endogenous fly kinases produce a basal level of tau phosphorylation of the PHF1 and AT270 epitopes. Phosphorylation of the AT8 epitope appears to be very specific for human GSK3β in the eye. Moreover, phosphorylation of the soluble and insoluble tau fraction was measured *in vivo* in the fly line co-expressing human tau and GSK3β. Upon GSK3β expression, an increased phosphorylation was observed at PHF1 and AT270 epitopes in the soluble and insoluble fractions of tau, as seen in S2 cells. In particular, phosphorylation of S396 and S404 underwent a very large increase of about 40 fold in the soluble fraction and about 20 fold in the insoluble fraction. On the other hand, the increase of phosphorylation of T181 was measured to be around 3 fold in the soluble fraction and 4 fold in the insoluble fraction. In contrast, phosphorylation of the AT8 epitope does not change significantly upon GSK3β expression, differently from the data obtained from the *in vitro* experiments.

These results lead to the conclusion that the enhancement in tau-mediated eye degeneration caused by GSK3β *in vivo* might be due to increased phosphorylation of

human tau in the soluble and insoluble forms. More importantly, S396, S404 and T181 appears to be key sites to be targeted for GSK3β phosphorylation of tau and they might be involved in generating more toxic forms of human tau in the fly eye. Interestingly, previous *in vitro* evidence show that phosphorylation of S396 and S404 is primarily responsible for the functional loss of tau-mediated tubulin polymerisation, suggesting a possible role in initiating the destabilisation of microtubules (Evans et al, 2000). These results possibly suggest that detachment of human tau from microtubules due to phosphorylation by human GSK3β causes toxicity in the fly eye.

4.3.3 Effects of tau point mutations on GSK3β-mediated toxicity

Multiple phosphorylation or dephosphorylation events located in functionally regions of tau could cause the protein to alter its physiological function and become toxic. In order to modulate the toxicity generated by human kinases in the fly eye, some S/T residues on human tau were mutated in alanines to inhibit phosphorylation. In *Drosophila* tauopathy models, the role of phosphorylation by endogenous kinases at SP/TP sites on human tau has been proved to play a critical role in tau toxicity in flies. However, no-single phosphorylation residue seems to play a dominant role in controlling tau toxicity generated by endogenous kinases (Steinhilb et al, 2007a; Steinhilb et al, 2007b). Since my thesis focuses on the ability of human kinases to enhance human tau-mediated degeneration in flies, single phospho-residues were mutated to alanines to see whether additional toxicity generated by the introduction of human kinases could be modulated and mapped to individual sites.

The choice of the residues to mutate was driven by the results obtained from previous *in vitro* and *in vivo* experiments in this thesis and by the pathological relevance of the phosphorylation of those sites. Moreover, all mutated sites are phosphorylation targets of DYRK1A, a novel kinase found to be involved in tau hyperphosphorylation and suggested to prime GSK3β phosphorylation of tau (Liu et al, 2008; Woods et al, 2001). The mutations studied were S202A, T205A, T212A and S404A. In this study, phosphorylation of S202 and T205 of human tau was shown to increase in S2 cells transfected with human GSK3β, while no increase was seen in fly eyes expressing

human tau together with human GSK3β. Interestingly, S202 and T205 were also demonstrated to be substrates for phosphorylation of endogenous kinases in the eye but not in S2 cells. The inhibition of phosphorylation of S202 and T205 did not affect the eye phenotypic enhancement generated by GSK3β, PKA and DYRK1A.

Moreover, the novel kinase DYRK1A has been suggested to prime the activity of GSK3β on tau via phosphorylation of T212. In the course of this thesis not enough evidence was obtained to demonstrate an increase of tau phosphorylation of T212 by human GSK3β. However, in fly eyes a low level of phosphorylation of this site occurs by endogenous kinases. Not surprisingly, when T212 was mutated to alanine, the enhancement in degeneration generated by GSK3β, PKA, CK1δ or DYRK1A on wild type tau was not affected. The same effects occurred when human tau T212A was expressed together with GSK3β and DYRK1A. As a result, individual phosphorylation of human tau on S202, T205 and T212 by human kinases does not appear to contribute to the tau-mediated degeneration caused by human GSK3β in this fly model of tauopathy.

Tau phosphorylation was also inhibited on S404, which is consistently hyperphosphorylated in neurofibrillary tangles together with S396 and widely believed to be important players in pathological tau action (Goedert et al, 1994). Moreover, in this thesis, phosphorylation of S396 and S404 has also been shown to significantly increase upon human GSK3β expression compared to other epitopes and, therefore, they are thought to be important in conferring tau toxicity. Surprisingly, inhibiting phosphorylation of S404 increased the degeneration generated by human GSK3β on wild type tau. In addition, when phosphorylation was inhibited on S202, T205, T212 and S404 at the same time, the eye degeneration increased upon human GSK3β expression, as for the mutation of S404 alone. These results suggest that dephosphorylation or mutation of S404 plays a crucial role in increasing tau toxicity.

4.3.4 S404 of human tau contributes to confer toxicity via GSK3β

In physiological conditions, tau binds directly to microtubules promoting their assembly and regulating microtubule dynamic instability essential for many cellular functions, including neuronal axonal transport (Lindwall & Cole, 1984; Mandelkow et al, 2003). Indeed, tau phosphorylation plays a physiological role in regulating the affinity of tau for microtubules: tau binding to microtubules is promoted by dephosphorylation of tau, while tau detachment is promoted by phosphorylation of tau (Buee et al, 2000). In tauopathies the main pathogenic event responsible for neuronal loss is tau hyperphosphorylation, although the molecular mechanisms by which tau mediates cell death remain elusive (Ballatore et al, 2007). Two widely accepted hypotheses consider tau hyperphosphorylation to cause gain-of-function and loss-of-function pathogenic effects for neurons. Firstly, abnormal phosphorylation increases tau propensity to aggregate into oligomers or tangles gaining a toxic function and leading to cytotoxicity (Ballatore et al, 2007). However, it has also been suggested that tau tangles may not be necessary for tau-mediated cell death since neurodegeneration can occur in the absence of tau aggregation *in vitro* and *in vivo* (Kambe et al, 2011; Wittmann et al, 2001). Alternatively, the tau-mediated neuronal cell death in tauopathies might be caused by a tau loss-of-function in the regulation of microtubule dynamic instability (Iqbal et al, 2009). Indeed, an abnormal regulation of tau phsophorylation might compromise its ability to promote microtubule assembly and therefore, to regulate microtubule dynamic instability (Amniai et al, 2009). Axonal transport mechanisms rely on microtubules as tracks for the transport of mitochondria and organelles to the synapse. Thus, the misregulation of microtubule dynamic instability disrupts axonal transport and can lead to cell cycle arrest followed by cell death (Morfini et al, 2009). In this case the misregulation of tau can occur due to pathogenic phosphorylation or dephosphorylation events that lead to aberrant microtubule detachment or attachment of tau respectively.

In this thesis, phosphorylation and dephosphorylation events of the S404 site alone were demonstrated to generate human tau toxicity in the *Drosophila* eye. Firstly, phosphorylation of the PHF1 epitope (which includes the S404 site) largely increases after human GSK3β expression and it is thought to be responsible for the eye degeneration caused *in vivo*. At the same time, inhibition of phosphorylation or

mutation of S404 enhances the eye degeneration caused by human GSK3β only. These results lead to the conclusion that S404 might be a crucial site mediating human tau toxicity in flies. Thus, a tight regulation of phosphorylation and dephosphoryaltion on this site might be required to maintain the physiological function of tau.

The model here proposed has been confirmed also by other *in vitro* and *in vivo* studies. Indeed, tau phosphorylation at S396 and S404 has been shown to be responsible for the impairment of microtubule assembly and dissociation of tau from microtubules leading to the disruption of the microtubule transport system *in vitro* (Evans et al, 2000; Kiris et al, 2011). Finally, in the cerebral cortex and hippocampus of transgenic mice with increased phosphorylation of S396 and S404 of human tau has been suggested to detach tau from microtubules and disturb the synaptic function leading to synapse loss (Kambe et al, 2011). Interestingly, phosphorylation of S404 *in vitro* has been shown to have a dominant effect on microtubule stability among the multiple phosphorylated sites (Kiris et al, 2011). On the other hand, cell culture studies have shown that inhibition of GSK3β leads to a decrease of phosphorylation of several proline-dependent sites, including S404. Tau dephosphorylation resulted in the inhibition of anterograde transport, while the mitochondrial and organelle transport was largely intact in cells containing phosphorylated tau (Tatebayashi et al, 2004). As a result, tau phosphorylation by GSK3β has been shown to regulate the anterograde transport of organelles and mitochondria (Tatebayashi et al, 2004). *In vivo* studies using *Drosophila* have also demonstrated that multiple mutations in human tau, including S404A, lead to increased tau toxicity upon shaggy expression. This increased toxicity was attributed to a pathogenic detachment of human tau from microtubules which altered the regulation of microtubule assembly leading to increased toxicity (Chatterjee et al, 2009).

In conclusion, it is possible that phosphorylation or dephosphorylation of tau at defined single sites cause phosphorylation-dependent conformational changes of tau or toxic effects on the dynamics of microtubule assembly that generate toxicity. In particular, the regulation of phosphorylation of S404 might play a crucial role in maintaining the physiological function of tau.

CHAPTER 5
Investigating the toxicity of different tau isoforms

5.1 Introduction

5.1.1 Tau isoforms and tau mutations in disease

Tau exists in a variety of isoforms and a number of mutations in tau are associated with disease. In adult human brains alternative splicing generates six tau isoforms ranging from 352 and 441 amino acids in length. These tau isoforms differ by the presence of none, one or two N-terminal inserts and 3 or 4 C-terminal repeats (Figure 1.1). The N-terminal inserts mediate the interaction between tau and the neural plasma membrane, other cytoskeletal elements and cytoplasmic organelles, while the C-terminal repeats are involved in tau binding to microtubules (Andreadis, 2005; Buee et al, 2000). During adulthood the majority of tau isoforms contain a single N-terminal insert (1N), while the ratio of 3R:4R isoforms is approximately 1 (Buee et al, 2000). In the previous chapters, I investigated the toxicity of the full-length tau protein (2N4R) containing two N-terminal domains and four C-terminal repeats. However, it is possible that different tau isoforms contribute differently to toxicity. Thus, I extended the assay presented in this thesis to see whether different tau isoforms exhibit different ability to confer toxicity.

Tauopathies are a collection of neurodegenerative diseases characterised by the deposition of insoluble intra-neuronal aggregates of tau, including AD, Pick's disease, FTDP-17, PSP and CBD (Dickson, 1997; van Slegtenhorst et al, 2000). In a minority of cases, genetic mutations have been identified in the tau gene that are responsible for the formation of toxic variants of tau responsible for the neurodegeneration underlying the tauopathies. Mutations in the tau gene can have multiple effects including alteration of microtubule assembly, promotion of tau filament formation, alteration of tau mRNA splicing and isoform expression (Hutton et al, 1998; Poorkaj et al, 1998; Spillantini et al, 1998). In particular, FTDP-17 patients carry autosomal-dominant mutations in the tau gene responsible for the formation of abundant tau inclusions in neurons (Hutton, 2001; Hutton et al, 1998). The types of mutations found in the coding region of tau include missense, deletion and silent mutations. These are mostly located in the microtubule-binding region, these mutations lead to a reduction in the affinity of tau for microtubules (Dayanandan et al, 1999). In contrast, five mutations are present in the intronic regions and they mainly affect the mRNA splicing of exon 10. As a

consequence, an increased expression of tau carrying four C-terminal repeats occurs together with the formation of more tau deposits (Hong et al, 1998). Moreover, genome-wide association analysis of PSP and CBD patients have shown that mutations in the tau gene also constitute a genetic risk factor for the development of these pathologies, in particular the MAPT H1 haplotype (Di Maria et al, 2000).

The majority of FTDP-17 missense mutations disrupt tau affinity and binding capacity to microtubules as well as reducing the ability of tau to polymerise tubulin (Dayanandan et al, 1999; Hasegawa et al, 1998; Hong et al, 1998; Spillantini & Goedert, 1998). Moreover, since the interaction between tau and microtubules is disrupted, the level of unbound tau in neurons increases, accelerating the formation of tau aggregates (Goedert et al, 1999; Nacharaju et al, 1999). In addition in some cases, missense mutations also decrease tau degradation by calpain resulting in an increased level of tau available for aggregation (Yen et al, 1999). Among the missense mutations leading to FTDP-17, only three affect all tau isoforms and are localised outside exon 10: G272V in exon 9, V337M in exon 12 and R406W in exon 13. These three mutations cause a neuronal pathology characterised by straight filaments composed of all six human tau isoforms, similar to the PHFs and straight filaments observed in AD (Barghorn et al, 2000; Crowther & Goedert, 2000).

Cell culture studies have demonstrated that the R406W mutation markedly reduces the ability of tau to bind microtubules and to promote their assembly when compared with other tau mutations or wild type tau proteins (Dayanandan et al, 1999; Hong et al, 1998; Pérez et al, 2000). Also, *in vivo* experiments showed that the R406W mutation on tau might induce a conformational change of the microtubule-binding domain leading to a reduction of microtubule-binding and assembly (Delobel et al, 2002; Zhang et al, 2004). Moreover, the tau gene mutation R406W alters the phosphorylation state of tau contributing to the formation of tau aggregates. Indeed, tau isoforms carrying this mutation exhibit a low level of phosphorylation in cells compared to the other variants (Dayanandan et al, 1999; Miyasaka et al, 2001; Sahara et al, 2000; Vogelsberg-Ragaglia et al, 2000). In particular, R406W decreases phosphorylation by GSK3β on S/T sites, including S195, S199, T231, S235, S396, S400 and S404 (Connell et al, 2001; Pérez et al, 2000). All together these defects cause an impaired axonal transport and tau accumulation into filamentous inclusions that eventually lead to axonal degeneration

(Zhang et al, 2004). The R406W mutation on tau has occurred as an independent event in several families leading to a slow rate of disease progression lasting up to 25 years, compared to an expected duration of illness of 10 years (Rademakers et al, 2003).

5.1.2 Using *Drosophila* to model the toxicity of different tau isoforms and to study the R406W tau mutation

Several *Drosophila* models of tauopathy have been created in the past decade recapitulating the progressive accumulation of insoluble tau and extensive neurodegeneration of human tauopathies. However, the discrepancy among these transgenic models is the use of different human tau isoforms and mutated forms of these that might behave differently in the fly as well as during disease in humans. There is a preference in using the 4R tau isoforms in *Drosophila*, especially 0N4R and 2N4R tau, for their completeness in the number of microtubule-binding repeats present. Moreover, *Drosophila* models of tauopathy have previously addressed the role of the FTDP-17 associated mutation R406W, which appears to confer high toxicity to human tau in flies (Nishimura et al, 2004; Wittmann et al, 2001).

Wittmann et al (2001) showed that human tau expression in the *Drosophila* CNS causes neurodegeneration and lifespan reduction without aggregate formation. In particular, this study demonstrated that the R406W and V337M mutations confer more toxicity compared to wild type human tau in flies. Moreover, tau phosphorylation in the CNS increased as the fly aged and accumulated in areas of degeneration. Additionally, it has been demonstrated that human tau carrying the R406W mutation confers more toxicity in the fly eye compared to wild type tau (Chau et al, 2006; Nishimura et al, 2004). No evidence of NFT-like aggregates has been shown, however phosphorylation of R406W tau was increased at S202, S262, S396 and S404 compared to wild-type tau. Moreover, the toxicity generated by R406W tau can be enhanced by *Drosophila* kinases via increased phosphorylation (Chau et al, 2006; Nishimura et al, 2004). PAR-1 kinase, the *Drosophila* orthologue of MARK, was identified as an early player in tau phosphorylation, initiating a temporally ordered series of additional tau phosphorylation events by downstream fly kinases, including cdk5 and GSK3β (Nishimura et al, 2004).

The human tau model described in the previous chapters expresses the 2N4R tau isoform and showed a considerably low level of toxicity in the fly eye compared to other published fly models (Chatterjee et al, 2009; Chau et al, 2006; Jackson et al, 2002; Shulman & Feany, 2003; Steinhilb et al, 2007a; Steinhilb et al, 2007b; Wittmann et al, 2001). This chapter aims to investigate the different toxicity levels of wild type and R406W tau and their differential interaction with human kinases. The *Drosophila* models previously reported were created via P-element mediated transgenesis, however in this study transgenic fly lines were generated using the phiC31-mediated transgenesis. This system allows the reproducibility of the genomic position of the transgene ensuring a standardised level of expression and genetic background. Different tau isoforms are compared for their ability to generate toxicity and to interact with human kinases relevant to disease in the fly eye.

5.2 Results

5.2.1 Exploiting the phiC31-mediated transgenesis to study the toxicity of different tau forms

The reported fly models of tauopathy aim to recapitulate the tau pathology by expressing different human tau isoforms. The tau isoform shown to confer more degeneration when expressed in the fly is the 0N4R tau carrying the R406W mutation (Nishimura et al, 2004; Wittmann et al, 2001). However, these fly models have been created using the P-element meditated transgenesis, which generates variability in expression levels and phenotype depending on the insertion site of the transgene (Chapter 3). Different human tau isoforms carrying the R406W mutation were tested for their ability to induce degeneration in the fly eye. Transgenic *Drosophila* lines were generated expressing human 2N4R tau R406W, 0N4R tau and 0N4R tau R406W. Using the phiC31-mediated transgenesis, the three human tau isoforms were inserted at the same genomic locus used by line 2N4Rtau#68A resulting in fly lines with the same genetic background and expression level of tau. Therefore, the tau-mediated toxicity in the fly eye is affected only by the tau isoform inserted avoiding any positional effect of the transgene. The aim of this experiment is to compare the ability of human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W to generate eye toxicity and to assess whether their interaction with the human kinases varies.

The transgenes were expressed in the eye using GMRGAL4. Gene expression was induced throughout development at 25°C and flies were collected between 0 to 5 days post-eclosion. The eye phenotypes were examined by light microscopy (Figure 5.1). As shown before, line 2N4Rtau#68A is characterised by a weak disarrangement of the ommatidial pattern compared to control (Figure 5.1A). When different isoforms of human tau are expressed in the fly eye, their resultant phenotypes exhibit similar characteristics to that of 2N4Rtau#68A (Figure 5.1B-D). The mutation R406W does not enhance the toxicity of tau in the fly eye compared to wild type human tau. Fly lines expressing human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W are characterised by a comparable weak disarrangement of the ommatidial pattern in the anterior part of the eye.

This result shows that tau isoforms differing for the number of N-terminal domains, including 2N4R tau and 0N4R tau, generate the same degree of degeneration when expressed at the same level and in the same genetic background in the fly eye. Moreover, in contrast with previous studies, the mutation R406W does not alter the toxicity conferred by either wild type 2N4R or 0N4R tau. Therefore, in a *Drosophila* context, the N-terminal domains of tau and the R406W mutation do not appear to participate in generating the tau-mediated eye degeneration.

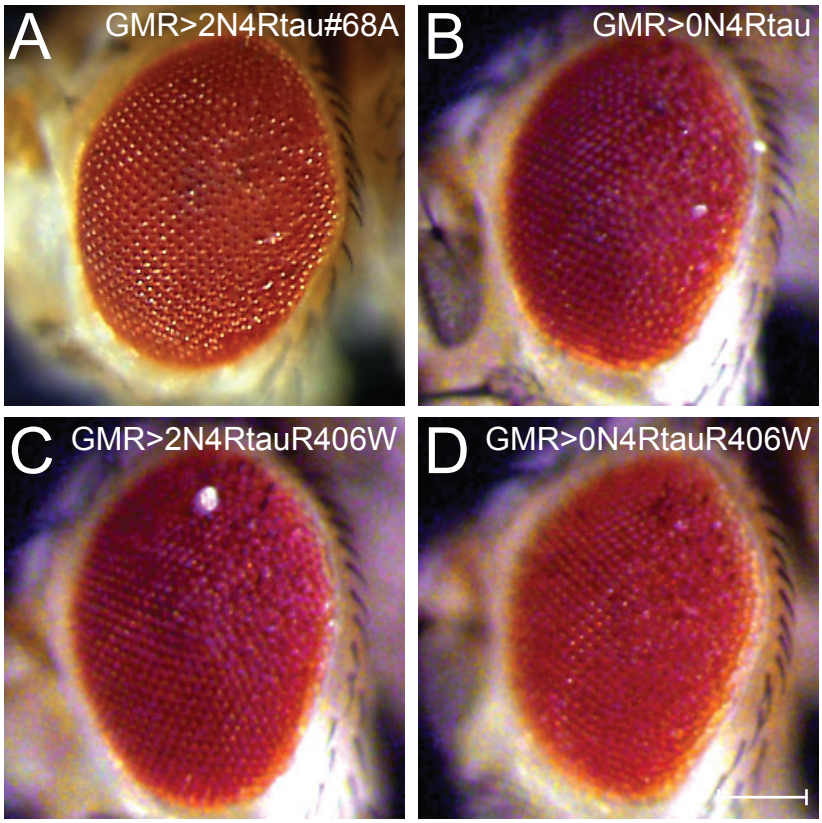

Figure 5.1 Expression of different tau isoforms from a phiC31-specific site causes similar levels of eye degeneration. Light micrographs of eyes from 0-5 days old flies. All transgenes were expressed in the fly eye using the GMRGAL4 driver. (A-D) Expression of the same low level of 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W cause the same level of weak ommatidial disruption. Scale bar: 100 μm. Dorsal is top and anterior is left.

The previous experiment shows that the fly lines expressing different tau isoforms do not differ in their ability to generate toxicity. The transcript levels of human tau were quantified via qPCR in order to confirm that they are equal in the lines created by phiC31-mediated transgenesis. The experimental design was planned as in previous experiments (Chapter 2 and 3). The results were standardised in order to ensure they could be compared with the values obtained in previous experiments. Human tau expression was controlled by the eye-specific driver GMRGAL4. Flies were raised at 18°C and after eclosion they were aged for three days at 25°C. The human tau transgenes were expressed alongside a UAS-CD8::GFP reporter gene using the same genetic driver. The spatial and temporal control of the reporter gene overlaps with that of the human tau transgene. The transcript level of human 2N4R tau in each transgenic *Drosophila* line was quantified using actin, GAPDH and the transcriptional factor eIF-4a as reference genes. The copy number of CD8::GFP was used to normalise for the actual number of eye cells expressing human tau. Flies carrying only the eye specific driver GMRGAL4 and reporter UAS-CD8::GFP were used as negative control (Figure 5.2). The P-element transgenic *Drosophila* lines carry different isoforms of the human tau transgene which are inserted at different genomic positions. Upon tau expression, the transgenic fly lines produce different levels of human tau transcript. To enable a better comparison between the different lines, the transcript levels were expressed in percentage relative to the highest mean. The fly lines expressing human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W inserted in position 68A using the phiC31-mediated transgenesis exhibit a statistically non-significant difference in the levels of transgene expressed. This result is expected since the transgenes are inserted in the same genomic locus and the experimental conditions were equal for all lines. The transcript expression for human tau was measured also for a widely used fly model of tauopathy, originally created by Wittmann et al (2001). This fly line carries the 0N4R tau R406W transgene (line MF_0N4RtauR406W) inserted in a random genomic position and it is characterised by an increased tau-mediated toxicity in the eye compared to that seen in the lines created in this study (Wittmann et al, 2001). The qPCR result shows that line MF_0N4RtauR406W expresses a statistically significant higher level of human tau compared to the lines created in this study via phiC31-mediated transgenesis. In particular, the level of tau expression for line MF_0N4RtauR406W is approximately the double that seen for line 0N4RtauR406W.

The tau transcript levels seen for lines 2N4Rtau#1 and 3 (Chapter 3) were compared to the tau isoforms measured here. Interestingly, line MF_0N4RtauR406W expresses the same level of tau transcript as line 2N4Rtau#1 expressing the 2N4R tau transgene inserted in genome at random. Finally, as in figure 3.5 and 3.7, line 2N4Rtau#3 is characterised by a level of tau transcript that is statistically much higher than the other *Drosophila* tau lines examined here.

This evidence suggests that at the expression level generated by tau transgene insertions in 68A, *Drosophila* lines expressing 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W show weak tau toxicity. To examine whether the different isoforms behave similarly at higher expression levels, the line generated by Wittmann et al (2001) was examined. This line has been reported to exhibit stronger tau-mediated eye degeneration and it is characterised by a higher level of human tau transcript compare with the lines generated via phiC31-mediated transgenesis.

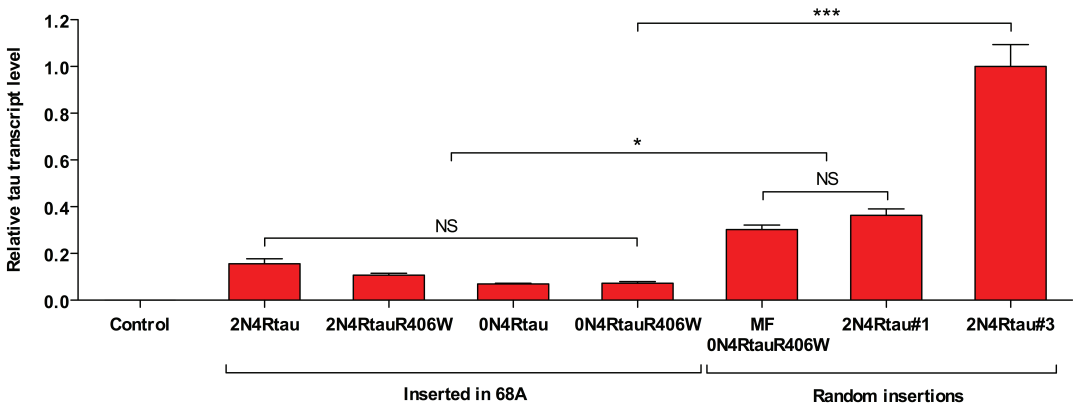

Figure 5.2 Variation of transcript levels of human tau in different *Drosophila* models of tauopathy. The transcript level of human tau was examined in flies expressing tau together with UAS-CD8::GFP using GMRGAL4. To enable comparison between samples, the transcript levels were expressed as relative copy number to the highest mean. Tau copy numbers were normalised to 3 reference genes and to UAS-CD8::GFP. Values correspond to the mean and SEM of 3 to 5 replicates from independent experiments. Asterisks indicate statistically significant differences of tau transcript. Control flies carrying the eye specific GMRGAL4,UAS-CD8::GFP driver show no human tau transcript expression. Transgenic flies carrying 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W transgenes inserted in the same genomic position show no significant difference in the tau transcript levels upon expression. The P-element inserted transgenes MF_0N4RtauR406W and 2N4Rtau#1 show the same high level of tau transcript levels. Transcript levels driven from 2N4Rtau#3 are significantly higher than that driven from all the other transgenes ($p<0.001$).

To examine the interaction between the different tau isoforms and human kinases, transgenic flies expressing human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W were combined with human GSK3β, PKA, CK1δ and rat DYRK1A in the fly eye. The eye degeneration is similar in flies expressing the different tau isoforms alone (Figure 5.3). When human GSK3β is co-expressed with human 2N4R tau, the eye degeneration phenotype is enhanced compared to human tau alone (Figure 5.3A2). Also, the eye degeneration of flies expressing other tau isoforms with human GSK3β is enhanced. The disarrangement of ommatidia is increased in the anterior part of the eye and the characteristic oval shape of the eye is altered compared to control. The degree of phenotypic enhancement via human GSK3β is comparable in flies expressing human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W (Figure 5.3A2, B2, C2 and D2). As in previous experiments shown in this study, human PKA co-expression with 2N4R tau does not lead to an enhancement of eye degeneration (Figure 5.3A3). Moreover, the tau lines carrying the different tau isoforms show no enhancement in eye phenotype when PKA is co-expressed (Figure 5.3B3, C3 and D3). Subsequently, co-expression of 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W together with human CK1δ does not affect the basal human tau-mediated eye degeneration caused in each line (Figure 5.3A4, B4, C4 and D4). It has been shown before in this study that rat DYRK1A co-expression with 2N4R tau causes a minor enhancement of human tau-mediated degeneration (Figure 5.3A5). Also, the tau-mediated eye degeneration caused by the different tau isoforms is mildly enhanced by expression of rat DYRK1A. The eye phenotypes are characterised by a minor enhancement of human tau-mediated degeneration comparable to the one seen for line 2N4Rtau#68A (Figure 5.3, panel B5, C5 and D5).

Human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W were also tested in their ability to alter the possible priming interaction of GSK3β by CK1δ or DYRK1A. The expression of different tau isoforms and the introduction of the R406W mutation appears not to change the enhancement of tau-mediated degeneration in flies expressing GSK3β and CK1δ or DYRK1A compared to that seen in the 2N4Rtau#68A background (Figure 5.3A6-7, B6-7, C6-7 and D6-7).

In conclusion, these results show that the human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W have a similar interaction with human kinases in flies.

Neither the N-terminal domains of tau or the R406W mutations appears to mediate the interaction of the individual human kinases GSK3β, PKA, CK1δ, and rat DYRK1A. In particular, the only human kinase showing an enhancement of tau-mediated toxicity in all lines is GSK3β.

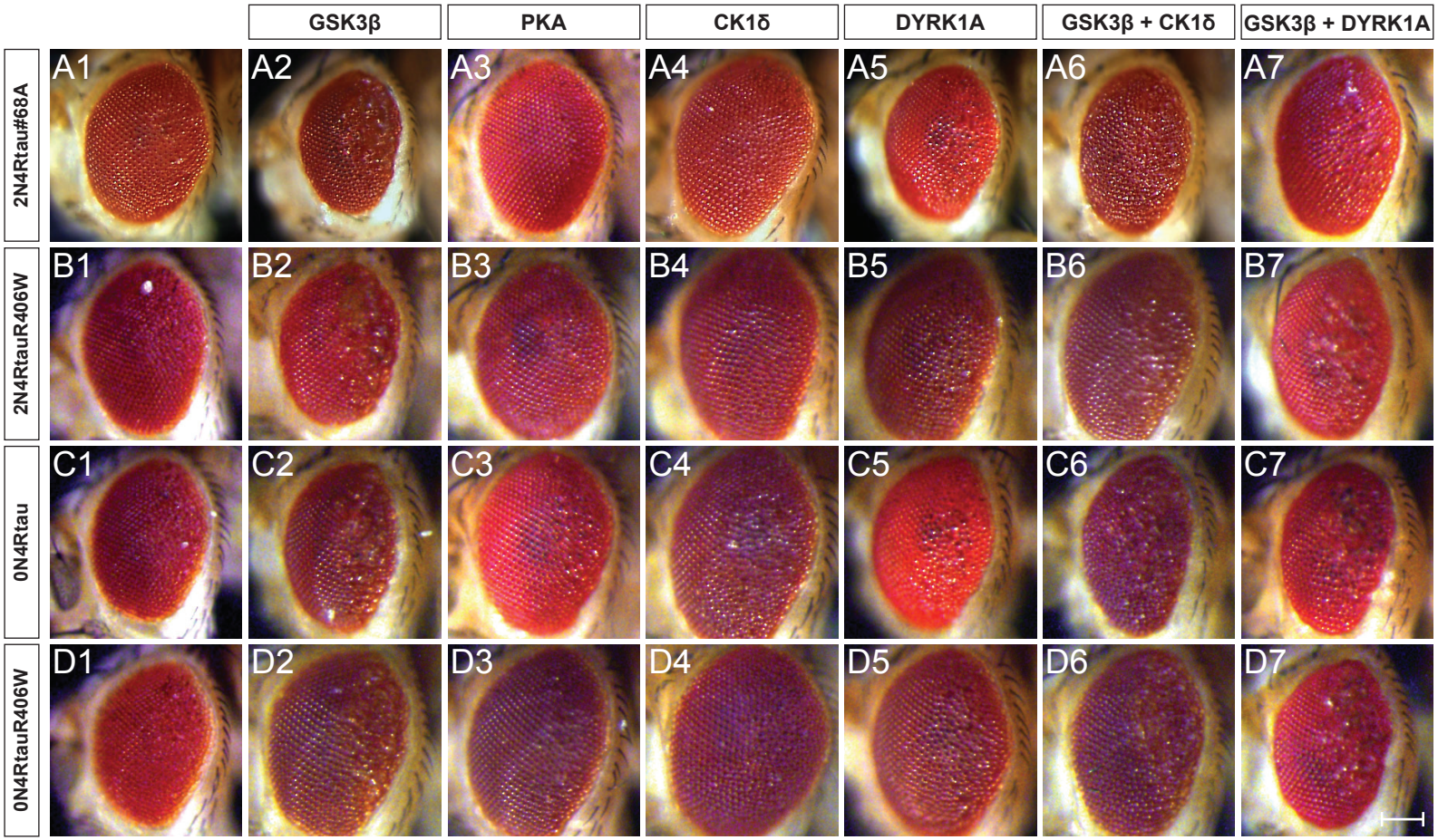

Figure 5.3 Different tau isoforms expressed at similar levels cause equal eye degeneration upon human kinase expression. Light micrographs of eyes from 0-5 days old flies. All transgenes were expressed in the fly eye using the GMRGAL4 driver. (A1, B1, C1 and D1) Expression of 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W cause weak ommatidial disruption. (B2-D7) The level of degeneration caused when the tau isoforms are expressed with human kinases is similar to that seen for 2N4R tau (A2-A7). Scale bar: 100 μm. Dorsal is top and anterior is left.

5.2.2 Understanding the importance of tau expression level in transgenic *Drosophila* models of tauopathy

In section 3.2.1 it has been shown that fly lines expressing a higher level of the transgene confer an increased tau-mediated toxicity. The ability of 2N4R tau and 0N4R tau R406W to induce eye degeneration was tested in fly lines with comparable expression of the transgenes. Lines 2N4Rtau#68A and 0N4RtauR406W express an equal amount of human 2N4R tau and 0N4R tau R406W respectively. In contrast, lines 2N4Rtau#1 and MF_0N4RtauR406W express approximately double the amount of transcript for human tau compared with the lines above. I investigated whether the interaction of the different tau isoforms with the human kinases varies when they are expressed at different levels in the fly eye.

The different tau constructs were expressed using the GMRGAL4 that drives the expression of the transgenes in the fly eye. Gene expression was induced throughout development at 25°C and flies were collected between 0 to 5 days post-eclosion. The eye phenotypes were examined by light microscopy (Figure 5.4). As shown above, line 2N4Rtau#68A and line 0N4RtauR406W are characterised by a weak disarrangement of the ommatidial pattern, which is enhanced by co-expression of human GSK3β (Figure 5.3A1-2 and D1-2). Line 2N4Rtau#1 expresses double the amount of 2N4R tau compared to line 2N4Rtau#68A. The eye phenotype caused by expression of human tau alone shows greater degeneration when 2N4Rtau is expressed from the insertion 2N4Rtau#1 (Figure 5.4B1). When expression is driven from 2N4Rtau#1 together with the kinases the degree of enhancement is similar to that seen when expression is driven from 2N4Rtau#68A with the same kinases. Human GSK3β and rat DYRK1A enhances the tau-mediated phenotype, while PKA and CK1δ do not have any effect (Figure 5.4B2-5). Interestingly, when co-expressed with 2N4Rtau#1, rat DYRK1A appears to cause a greater enhancement of eye degeneration compared to when expressed with tau from line 2N4Rtau#68A. In fact, after co-expression of high levels of 2N4R tau and rat DYRK1A the eye is characterised by the presence of several necrotic ommatidia not seen when driving tau from line 2N4Rtau#68A (Figure 5.4B5). In flies expressing tau from the transgene of line MF_0N4RtauR406W, the eye degeneration is greater compared to that seen for line 2N4Rtau#68A, 2N4Rtau#1 and 0N4RtauR406W (Figure

5.4D1). This result is similar to that seen in previous findings (Chau et al, 2006; Nishimura et al, 2004). Line MF_0N4RtauR406W was combined with the human kinases in order to see how they affect the toxicity of 0N4R tau R406W when expression is driven at higher levels. All kinases produced a greater enhancement of eye degeneration compared to that seen in previous experiments (Figure 5.4D2-7). Co-expression of human GSK3β together with MF_0N4RtauR406W causes a severe enhancement of eye degeneration characterised by the presence of large patches of necrotic ommatidia (Figure 5.4D2). Moreover, the human kinase PKA, that did not cause an increase in tau toxicity with other tau fly lines, enhances the human tau-mediated degeneration caused by MF_0N4RtauR406W in the eye. The eye is characterised by an extended disarrangement of ommatidia and the presence of several necrotic ommatidia (Figure5.4D3). However, the expression of human MF_0N4RtauR406W and CK1δ did not produce any enhancement of eye degeneration (Figure 5.4D4). Finally, rat DYRK1A strongly increased the eye degeneration caused by MF_0N4RtauR406W alone. In this case, the eye size is slightly reduced and large necrotic patches of ommatidia are present. The different tau isoforms were also tested for changes in toxicity when expressed with GSK3β together with CK1δ or DYRK1A. When human tau expression is driven at low levels in lines 2N4Rtau#68A and 0N4RtauR406W, CK1δ or DYRK1A do not appear to have a synergistic activity on tau with human GSK3β (Figure 5.4A6-7, C6-7). However, when human tau is expressed at higher levels, a synergistic interaction might be present between human GSK3β and rat DYRK1A. When 2N4R tau is expressed from line 2N4Rtau#1, GSK3β and DYRK1A together enhance the eye degeneration phenotype as revealed by an increase of necrotic ommatidia and eye depigmentation (Figure 5.4B6-7). Moreover, when these two kinases are co-expressed in line MF_0N4RtauR406W, the eye exhibits a high number of necrotic ommatidia, a strong depigmentation and a reduction in size (Figure 5.4D6-7).

In conclusion, when expressed at a higher and equal level, the 0N4R tau R406W appears to generate more toxicity in the fly eye compared to 2N4R tau. In contrast, at low levels of expression 0N4R tau R406W and 2N4R tau exhibit the same weak level of toxicity. Interestingly, the fly lines expressing higher tau levels were both generated through P-element mediated transgenesis, therefore it might be possible that endogenous genes are affected by the insertion of the transgene and affect the eye

toxicity. Moreover, this evidence shows that in fly models where human tau is highly expressed, the interaction with human kinases results in a more evident phenotypic enhancement of eye degeneration. In particular, tau-mediated toxicity was enhanced by human kinases, which did not show an interaction with human tau at low expression levels.

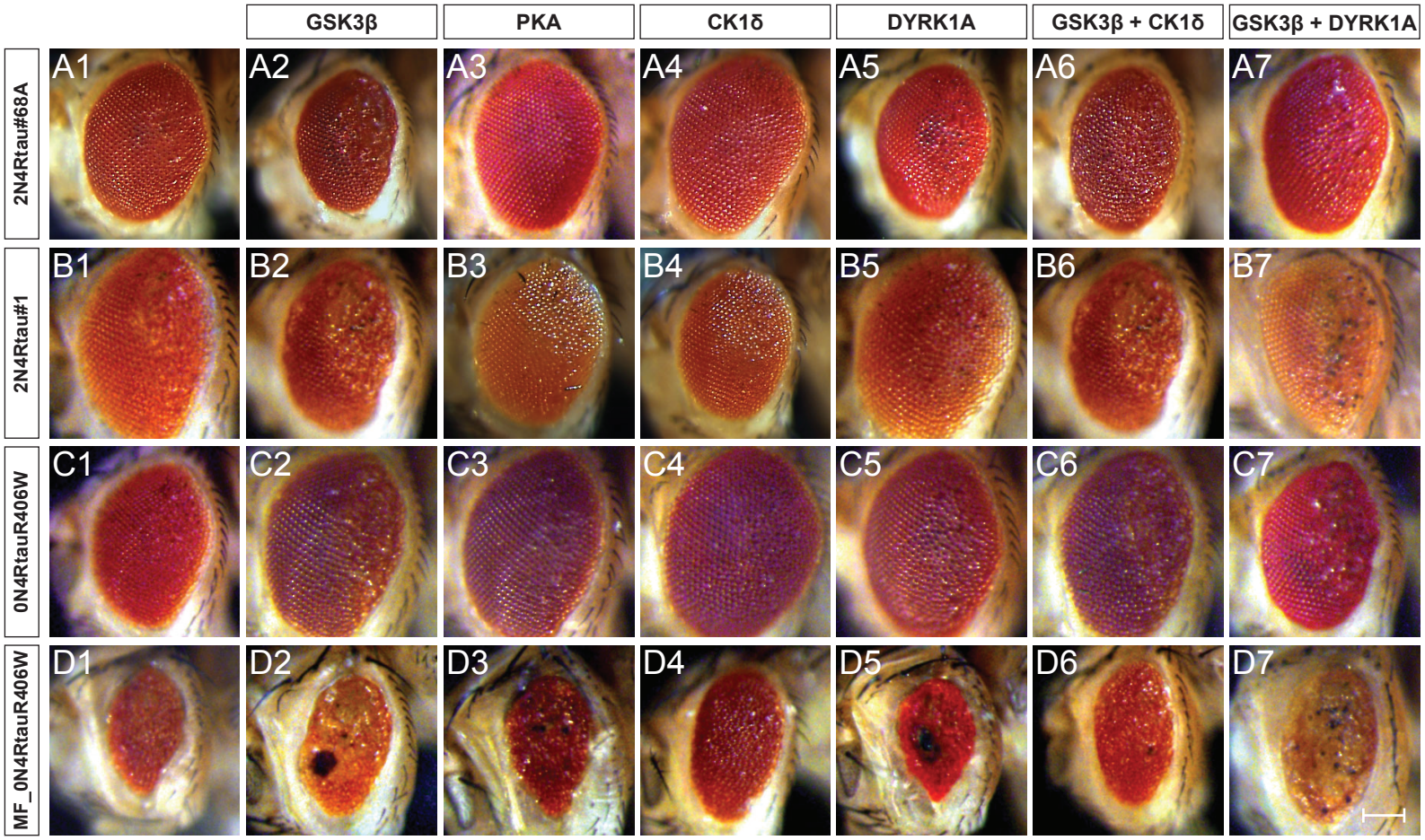

Figure 5.4 0N4R tau R406W exhibits a greater toxicity than 2N4R when expressed at higher expression levels. Light micrographs of eyes from 0-5 days old flies. All transgenes were expressed in the fly eye using the GMRGAL4 driver. (A1-D8) Expression of different levels of 2N4R tau and 0N4R tau R406W cause a variation in enhancement of eye degeneration when kinases are co-expressed. MF_0N4RtauR406W shows the strongest enhancement when co-expressed with kinases. Scale bar: 100 μm. Dorsal is top and anterior is left.

Increased expression levels of tau isoforms reveal differences in their ability to confer toxicity in the fly eye. To test whether an increased amount of tau isoforms or the mutant forms expressed from the phiC31-mediated insertions can generate a different level of toxicity I increased the level of expression in the lines by doubling the copy number.

Human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W were driven in flies carrying one or two copies of the transgene in position 68A. The aim of this experiment is to compare the eye degeneration caused by fly line expressing low or increased levels of human tau to determine whether the presence of the N-terminal domains or the R406W mutation contribute to more toxic forms of tau. Human tau was expressed in the fly eye using the GMRGAL4 driver. Gene expression was induced throughout development at 25°C and flies were collected between 0 to 5 days post-eclosion. The eye phenotypes were examined by light microscopy (Figure 5.5). As shown before, when one copy of human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W is expressed, the resulting eye phenotype is comparable. It is characterised by a weak disarrangement of the ommatidial pattern in the anterior part of the eye (Figure 5.5A-D). Subsequently, human tau expression was increased doubling the number of copies of transgenes present. At higher expression levels, the different tau isoforms cause a greater level of degeneration in the fly eye compared to the same isoforms expressed at lower levels. Indeed, the ommatidia disarrangement is increased and extends on the whole eye surface in the flies with a high tau level. Moreover, flies expressing the 0N4R tau and 0N4R tau R406W are characterised by a mild loss of tissue on the bottom anterior part of the eye (Figure 5.5E-H). However, when compared between each other, these transgenic lines expressing different tau isoforms generate the same degree of eye phenotype at high expression levels.

In conclusion, human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W were demonstrated to confer the same level of toxicity in the fly eye when expressed from a conserved site. This result is in contrast with previous studies showing a higher toxicity of 0N4R tau R406W compared to the wild type form in the fly eye (Nishimura et al, 2004; Wittmann et al, 2001). The fly models previously reported were created via standard P-element transgenesis allowing a random insertion of the transgene insertion in the fly genome. For this reason, an alteration of the degenerative phenotype due to

the genomic position of the transgene cannot be excluded and subtle differences in expression level. In this study, using the phiC31-mediated transgenesis, the toxicity of different tau isoforms was compared avoiding any positional effect of the transgene. The R406W mutation might not affect tau toxicity in the fly eye.

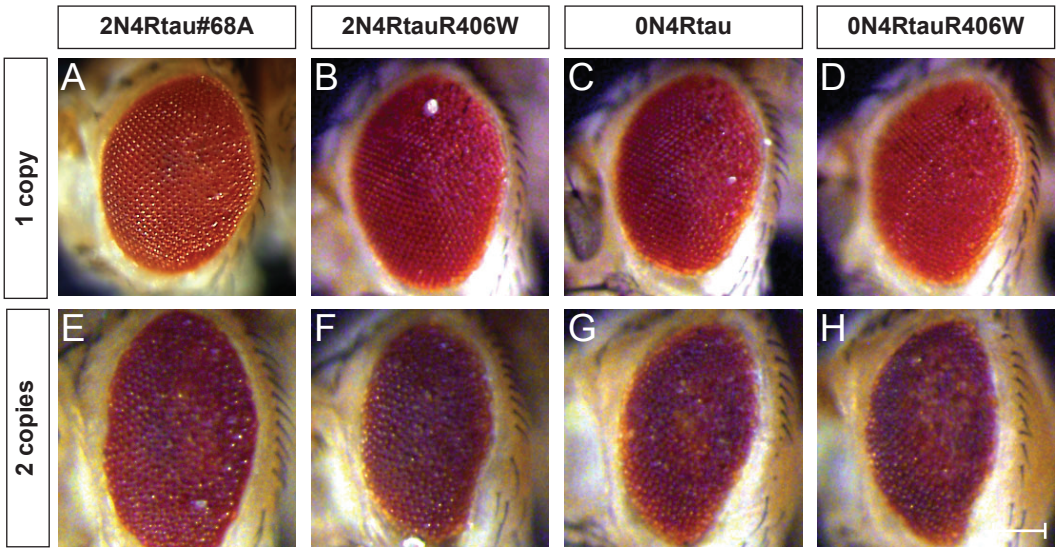

Figure 5.5 Tau isoforms do not exhibit a different level of toxicity when inserted in the same genetic background in *Drosophila*. Light micrographs of eyes from 0-5 days old flies. All transgenes were expressed in the fly eye using the GMRGAL4 driver. (A-D) One copy of 2N4R tau , 2N4R tau R406W, 0N4R tau and 0N4R tau R406W tau is expressed. (E-H) The tau-mediated eye degeneration is enhanced when the two copies of the transgenes are expressed. The tau isoforms do not display different levels of toxicity. Scale bar: 100 μm. Dorsal is top and anterior is left.

The toxicity generated by 0N4R tau R406W appeared to be increased by human kinases compared to 2N4R tau expressed at the same level (Figure 5.4). Thus, I investigated whether the tau isoforms and their mutated forms show different phosphorylation by human kinases in *Drosophila*.

In *Drosophila* S2 cells the interaction of human tau with human GSK3β causes a mobility shift of tau probably due to phosphorylation. To investigate the phosphorylation pattern of different tau isoforms, human GSK3β was transfected in S2 cells together with human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W. Expression of human tau was driven using an inducible UAS-GAL4 system activated by adding copper sulphate. Full-length human GSK3β, carrying a C-terminus myc-tag, was expressed using an actin promoter. Protein expression occurred at 25°C. Expression of wild type and mutated human 2N4R tau was induced for five hours while human GSK3β was constitutively expressed. Expression of human tau and GSK3β was successfully detected by western blot (Figure 5.6A). The phospho-independent tau antibody recognises the total level of tau expressed. Human GSK3β was detected using an anti-myc antibody and its mobility appeared to be at the expected molecular weight (60 kDa). Actin expression was used to control for protein loading. Control cells carrying an empty vector show only actin expression. Due to different levels of basal phosphorylation by endogenous kinases, wild type and mutated human tau were detected on the blot as multiple bands ranging from 60 kDa to 65 kDa for 2N4R tau and from 55 kDa to 60 kDa for 0N4R tau. The tau isoforms carrying the R406W mutation exhibit a multiple band pattern similar to wild type tau. When wild type and mutated tau was co-transfected together with human GSK3β, the mobility of all isoforms was reduced (Figure 5.6A). In order to monitor the phosphorylation level of wild type and mutated tau, the same samples were analysed with phospho-specific antibodies for human tau (Figure 5.6B). The phospho-specific antibodies chosen are PHF1 (pS396 and pS404), AT8 (pS202 and pT205) and AT270 (pT181). Control cells carrying an empty vector show no phospho tau expression. Phosphorylation at the PHF1 and AT8 epitopes was detected only in the samples where GSK3β was co-expressed together with tau. In contrast, the AT270 antibody recognises a basal level of phosphorylation of human tau by endogenous kinases. The amount of this epitope is strongly increased only when GSK3β is co-expressed.

These results suggest that 2N4R and 0N4R tau exhibit the same phosphorylation pattern on T181, S202, T205, S396 and S404. Moreover, the R406W mutation does not affect phosphorylation by endogenous kinases or human GSK3β on 2N4R and 0N4R tau as revealed by these phospho-specific antibodies.

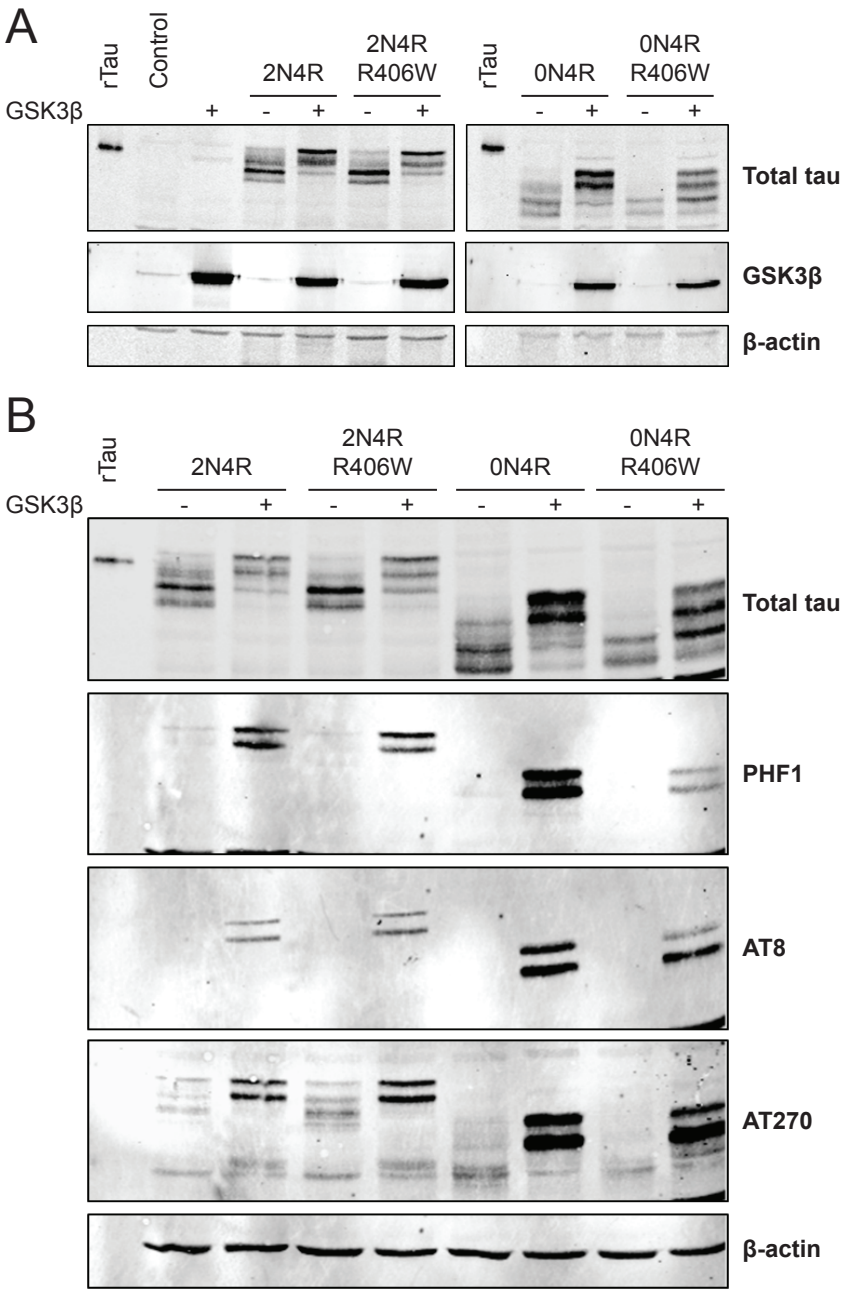

Figure 5.6 The phosphorylation pattern of GSK3β on human tau in *Drosophila* S2 cells does not vary with the tau isoform expressed. After transfection, human tau, GSK3β and actin expression were detected on western blot of S2 cells lysates. 2N4R tau, 2N4R tau R406W, 0N4R, 0N4R tau R406W where co-transfected with or without GSK3β. Recombinant human 2N4R tau (rTau) was loaded as positive control for tau and S2 cells transfected with an empty vector were used as negative control. Total tau was detected as multiple bands (60-70 kDa) using the phospho-independent antibody Dako. β-actin was used as loading control (42 kDa). (A) GSK3β was detected using an anti-myc antibody (60 kDa). (B) Phosphorylated human tau was detected using phospho-specific antibodies PHF1 (pS396 and pS404), AT8 (pS202 and pT205) and AT270 (pT181). There is no significant change in the phosphorylation pattern of the tau isoforms by GSK3β.

5.3 Discussion

Drosophila has been extensively used to model human tauopathies and investigate the interaction between human tau and endogenous fly kinases. The models have been created by driving expression of different tau isoforms and their mutated forms from sites inserted in the fly genome at random positions via P-element mediated transgenesis. The majority of the fly models created to date use the tau isoforms containing four C-terminal repeats, however some tau mutations have been shown to generate more toxicity. In particular, previous studies have been shown that human 0N4R tau carrying the FTDP-17 associated R406W mutation is characterised by an increased toxicity compared to wild type tau in *Drosophila* (Nishimura et al, 2004; Wittmann et al, 2001). The different tau isoforms used, levels of transgene expression and genetic background pose several difficulties in comparing results obtained in different research studies using the previous fly models of tauopathy. The phiC31-mediated transgenesis in *Drosophila* allows the insertion of transgenes in specific genomic loci (Bischof et al, 2007; Groth et al, 2004). Several transgenic *Drosophila* lines can be created carrying transgenes inserted in the same genomic position ensuring the same transgene expression levels and genetic background. This technique is useful to solve the issue related to variations of eye degeneration due to positional effect and expression levels of the human tau transgene.

This chapter proposed to use the phiC31-mediated transgenesis system to study the toxicity levels of different human tau isoforms and their mutated forms in *Drosophila*. In particular, tau isoforms containing none or two N-terminal domains and all four C-terminal repeats were tested. Moreover, the role of the FTDP-17 associated mutation R406W was also examined. Thus, transgenic fly lines were generated carrying human 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W inserted in the same genomic position (68A). These lines are characterised by the same genetic background and expression level of the transgene. When the toxicity of the tau isoforms and mutants was tested in the fly eye, a weak degeneration was observed in all lines. In contrast with the study by Wittmann et al (2001), the R406W mutation did not confer more toxicity to wild type tau when expressed in the fly eye. Moreover, the N-terminal domains did not appear to contribute to the tau-mediated degeneration either.

In addition, the different tau isoforms and their mutated forms were also tested for their ability to interact with human kinases in flies. The human kinase that confers greater toxicity to all tau forms is GSK3β. Human CK1δ and DYRK1A also weakly enhance the eye degeneration caused by tau, while PKA does not. The effect that these human kinases produced on the tau-mediated eye degeneration was comparable in flies expressing wild type and mutated human tau. Moreover, the ability of GSK3β to phosphorylate 2N4R tau, 2N4R tau R406W, 0N4R tau and 0N4R tau R406W was compared in *Drosophila* cell culture. The phosphorylation pattern on T181, S202, T205, S396 and S404 was equal in all tau isoforms and their mutant forms. Thus, the presence of N-terminal domains or of the R406W mutation on tau does not affect tau toxicity when these human kinases are also present. The effect of human kinases on tau-toxicity was also tested using the 0N4R tau R406W insertion line used in Wittmann et al (2001). In this case, human GSK3β, CK1δ, PKA and DYRK1A all cause a greater degeneration phenotype when combined to 0N4R tau R406W in flies.

The analysis of the tau transcript level confirmed that the expression level of human tau in the flies created with the phiC31 system is significantly lower compared to the fly line used in the publication by Wittmann et al (2001). In chapter 3, many *Drosophila* lines expressing wild type human 2N4R tau were generated expressing different levels of the transgene. One of these fly lines (2N4Rtau#1) exhibit the same level of tau expression compared to the line used in the report by Wittmann et al (2001). The toxicity generated by 0N4R tau R406W alone and combined with human kinases was higher than wild type 2N4R tau in fly lines expressing the same increased amount of tau transcript. This chapter shows that the N-terminal domains and the R406W mutation do not contribute to generate tau-toxicity in flies when tau is expressed at low levels. In contrast, at higher levels of expression, the absence of N-terminal domains and the presence of the R406W mutation on tau were shown to produce more toxicity than wild type tau containing two N-terminal domains. The reason for this difference might be related to the expression level of the transgene. A minimum level of transgene expression might be needed in order for changes in toxicity to be identified. In addition, the transcript analysis also revealed that the fly lines created via phiC31-mediated transgenesis express approximately half of the tau transcript expressed by the R406W created by Wittmann et al (2001). When the transgene level was increased by expressing two copies of the tau gene, the degenerative phenotype observed in the eye

was enhanced compared to when tau was in single copy. However, also using these conditions, the R406W mutation did not appear to confer an increased toxicity compared to wild type tau. Interestingly, the R406W mutation on tau in FTDP-17 patients leads to a late onset and long duration of the disease compared to patients that carry other mutations (Rademakers et al, 2003). On the basis of this observation, a *Drosophila* model of FTDP-17 carrying the R406W mutation might be expected to present a low level of tau toxicity and increased viability compared to flies expressing wild type tau.

In conclusion, using the phiC31-mediated transgenesis it is possible to generate *Drosophila* lines carrying transgenes inserted in the same genomic position. One of the major advantages of this system is the equal genetic background of the transgenic flies created and the absence of positional effects of the insertion on toxicity. When comparing transgenic fly lines generated via phiC31-mediated transgenesis, the only difference is in the transgenic insertion carried in the genome. Although the phiC31-mediated transgenesis allows a reproducible method of transgene insertion in *Drosophila*, the expression level of the transgene is low compared to flies generated via P-element mediated transgenesis. Using this system, the FTDP-relevant mutation R406W on tau was demonstrated to confer a similar level of toxicity as wild-type tau in the fly eye. These findings are in contrast with previous studies and the reason for this discrepancy may lie on the different methods used to produce the transgenic fly models. The fly line generated by Wittmann et al (2001) might present an increased tau-mediated toxicity because of positional effects caused by the random insertion of the R406W tau transgene in the fly genome.

CHAPTER 6
Discussion and future perspectives

Tauopathies are a collection of neurodegenerative diseases that include Alzheimer's disease, FTDP-17, Pick's disease, PSP and CBD. AD is the common type of senile dementia whose symptoms include decline in memory, confusion, disorientation, impaired communication and loss of motor function (Walsh and Selkoe, 2004). The other disorders are grouped in the FTLDs-tau because of a progressive degeneration affecting mainly the frontal and temporal lobes of the brain. Typical symptoms include changes in personality and behaviour as well as difficulty with language (Josephs et al, 2011; Neary et al, 1998). The main pathological feature that contributes to the neurodegenerative process in tauopathies is the formation of tau aggregates containing high levels of hyperphosphorylated tau (Alonso et al, 2001; Ballatore et al, 2007; Lauckner et al, 2003). Indeed, the normal physiological role of phosphorylation regulating tau function may be disrupted during disease by a series of aberrant phosphorylation events. This contributes to the formation of tau aggregates precipitating in neurons and leading to toxicity (Alonso et al, 2001; Ballatore et al, 2007; Lauckner et al, 2003). Tau aggregation reduces the normal microtubule-binding activity of tau resulting in the depolymerisation of microtubules (Amniai et al, 2009; Ballatore et al, 2007; Iqbal et al, 2009). The destruction of the microtubule network affects axonal transport and synaptic integrity (Morfini et al, 2009; Terry et al, 1991). These alterations, together with the impairment of cellular functions due to the formation of insoluble tau aggregates, lead to cell death (Ballatore et al, 2007; Iqbal et al, 2009).

The human kinases responsible for tau hyperphosphorylation *in vivo* have not been fully identified yet and little is known about their individual importance to tau phosphorylation during tauopathies. It is still debated whether the pathological tau kinases correspond to those that normally regulate the physiological function of tau or whether new kinases participate to generate the aberrant tau phosphorylation in disease. When identified, the pathological tau kinases can be used as targets for therapies to slow the degenerative process during disease. Reducing tau phosphorylation through inhibition of specific protein kinases is a promising approach to reduce tau aggregation and associated neuronal loss (Lau et al, 2002).

My thesis aimed to establish a *Drosophila* model of tauopathy in order to monitor tau toxicity and to identify the most pathogenic tau kinases. *Drosophila* is a widely used animal model to study tauopathies as it shows key features of the human disorders

including human tau-driven neurotoxicity. Previous work has investigated the interaction of human tau with endogenous *Drosophila* kinases (Jackson et al, 2002; Khurana et al, 2010; Shulman & Feany, 2003; Steinhilb et al, 2007a; Steinhilb et al, 2007b; Wittmann et al, 2001). Importantly and in contrast with previous studies, the model created here allows the study of the contribution of individual human kinases and their phosphorylation sites to human tau toxicity in *Drosophila*. I believe this approach is more disease relevant since the transgenic fly model is used to study the interaction between human proteins in contrast with studies where human tau and endogenous *Drosophila* kinases were used. Moreover, human kinases may confer toxic phosphorylation sites in human tau that are not targeted by endogenous kinases. Therefore, this system can reveal additional phosphorylation sites on tau that confer greater toxicity when human kinases are expressed.

6.1 Transgenesis methods to establish *Drosophila* models of tauopathy

Another aspect that diversifies the *Drosophila* model created in this thesis with those previously used concerns the method of transgenesis used to generate the transgenic fly lines. Previous fly models of tauopathy were created using standard P-element mediated transgenesis that allows the integration of the transgene in random chromosomal sites of the *Drosophila* genome (Rubin & Spradling, 1982; Spradling & Rubin, 1982). This method leads to the generation of transgenic fly lines characterised by different genetic backgrounds and by a variable expression level of the transgene that cannot be reproduced. This feature poses difficulties when comparing the toxicity of different tau constructs since the phenotype can be significantly affected by the site of P-element insertion. In this thesis I investigated the level of human tau toxicity in several fly lines produced with this method of transgenesis. I found that the same 2N4R tau transgene inserted in different chromosomal sites can be expressed at significantly different levels and ultimately lead to variations in phenotype intensity in the *Drosophila* eye. The human tau-mediated toxicity corresponds to the level of transcription expression of the human transgene. In conclusion, the genomic position of the transgene has a strong influence on the intensity of phenotype produced since it affects its transcription levels

in *Drosophila*. When investigating the phenotypic effects of constructs carrying different tau isoforms or mutated forms of those, it is ideal to standardise the transgene expression level and the genetic background of the fly models created to allow a better comparison. For this reason a novel method of transgenesis, the phiC31-mediated transgenesis, was used in this thesis to allow the integration of transgenes in specific and reproducible genomic loci (Bischof et al, 2007; Groth et al, 2004). In this way, the *Drosophila* lines created here carry the human transgenes inserted in the same genomic locus and so have the same genetic background and a reproducible expression level. The phiC31 system overcomes the phenotypic variations due to positional effect of the transgenes, thus the resulting phenotype is affected only by the type of transgene inserted.

The *Drosophila* lines expressing human 2N4R tau produced a degenerative phenotype in the eye as previously demonstrated (Jackson et al, 2002; Nishimura et al, 2004; Wittmann et al, 2001). Unexpectedly, the lines created using the phiC31-mediated transgenesis exhibited a weak eye phenotype and a lower transgene expression compared to the lines created using the P-element mediated transgenesis. One of the reasons for the weaker expression could be related to the chromosomal sites of transgene insertion chosen for the phiC31-mediated system. The fly lines available for site-specific transgenesis have the attP attachment sites located in non-coding genomic positions that might be refractory to high levels of transcription due to repressive effect of the surrounding chromatin (Groth et al, 2004; Markstein et al, 2008). It has been shown that the positional effect can also cause a large variation in level of transgene expression from tissue to tissue. In light of this, I chose a site of insertion that has been characterised by high expression levels in the CNS (Markstein et al, 2008). The lower level of expression was unexpected but allowed the opportunity to identify factors that could increase the level of degeneration.

It would be useful to improve the *Drosophila* model of tauopathy created here using the phiC31-mediated transgenesis and increasing the expression level of the transgene and therefore the phenotype generated. One possible remedy would be to engineer the transgenes to include insulators, stretches of DNA that block the effects of neighbouring enhancers and silencers of transcription as well as encroaching heterochromatin (Gaszner & Felsenfeld, 2006). Recently Markstein et al (2008) found that in transgenic

Drosophila the insertion of a gypsy retrovirus insulator flanking the transgene increases the transcriptional activity of many attP sites using the GAL4 system. With this technique, the expression of human tau in the model presented here could be expressed at high levels and produce a greater phenotype in the eye.

6.2 Human GSK3β enhances tau toxicity in *Drosophila* via site-specific phosphorylation

My thesis aimed to establish a *Drosophila* model of tauopathy and to identify the human kinases that produce toxic forms of tau. An advantage of using a fly line expressing human tau with an initial low level of degeneration is the possibility that identification of enhancers of the tau-mediated toxicity could be easier. In contrast with previous studies in flies, human kinases were tested for their ability to enhance the human tau toxicity generated in the transgenic phiC31 *Drosophila* lines. Selected human kinases relevant to tau pathology were studied, including GSK3β, PKA, CK1δ and DYRK1A. Human GSK3β was found to generate the strongest enhancement of human tau toxicity, while the results for the other kinases did not lead to significant conclusions. This observation is confirmed by previous findings showing that GSK3β is thought to be one of the major pathogenic tau kinases involved during disease (Hanger et al, 1992; Lovestone et al, 1994a; Lucas et al, 2001).

The toxicity of human tau in flies can be enhanced by endogenous *Drosophila* kinase activity, which causes an increased phosphorylation of tau and alters its solubility properties (Chau et al, 2006; Jackson et al, 2002; Shulman & Feany, 2003). However, it has been shown that human tau-mediated neurodegeneration in flies occurs without neurofibrillary tangles characteristic of AD, but with the formation of insoluble pre-tangle tau aggregates (Chatterjee et al, 2009; Jackson et al, 2002; Wittmann et al, 2001). The human 2N4R tau expressed in the phiC31 fly line created in this study was found in the soluble and insoluble form probably due to phosphorylation by endogenous kinases on human tau as previously shown (Chatterjee et al, 2009; Chau et al, 2006). However, no correlation was found between the solubility levels of human tau and the degree of toxicity in the eye. Moreover, the additional activity of GSK3β did not alter tau

solubility although it enhanced its toxicity in the fly eye. An important observation from my thesis is that a change in tau solubility is not needed to increase its toxicity in flies. This result suggests that GSK3β might enhance tau toxicity via phosphorylation of specific sites that are not usually phosphorylated by endogenous fly kinases. It is possible the activity of multiple kinases could be required in order to alter tau solubility leading to the formation of the insoluble and aggregated tau forms found during human disease.

Since human GSK3β is found to enhance tau toxicity beyond the level achieved by endogenous kinases, this thesis attempted to identify whether single sites targeted by human GSK3β are responsible for the increased toxicity. I found that phosphorylation of tau on S396 and S404 is consistently increased by human GSK3β in *Drosophila* cell culture and in the eye compared to other epitopes. The increase in phosphorylation of these sites was of about 40 fold in the soluble fraction and about 20 fold in the insoluble fraction of tau extracted from fly eyes. Moreover, phosphorylation of T181 was found to be moderately increased by GSK3β, however it is also a substrate for endogenous kinases as well. Despite previous studies suggesting that no single phosphorylation residue seem to play a dominant role in controlling human tau mediated degeneration via phosphorylation by endogenous kinases, this thesis suggests that it is possible to identify a single site that contributes to tau toxicity mediated by a human kinase (Steinhilb et al, 2007a; Steinhilb et al, 2007b). In conclusion, S396, S404 and T181 appear to be key sites to be targeted by GSK3β phosphorylation and they might be involved in generating more toxic forms of human tau in the fly eye.

Further investigations would be needed to test the toxicity generated by human tau and GSK3β in *Drosophila* under conditions that more resemble human pathology. With the development of more sophisticated genetic tools for temporal control of gene expression, *Drosophila* is rapidly becoming a useful model organism for aging studies. For example, using the temperature-sensitive inhibitor of GAL4, $GAL80^{TS}$, transgene expression could be initiated only during adulthood in the fly eye to investigate the degeneration of photoreceptor neurons. In this thesis it was already shown that expression of human tau only during adulthood does not lead to neurodegeneration in the eye. However, it would be useful to investigate whether the increased tau toxicity generated by GSK3β could result in the degeneration of differentiated neurons in the

eye. In addition, to confirm the interaction between human tau and GSK3β in flies, the effect of the expression of these transgenes should be tested in another cell type, for example in motor neurons. The toxicity of tau can be assessed by using a climbing assay that measures the locomotor ability of flies. The loss of motor neuron function leads to a decline in climbing activity. Novel approaches have been recently developed to allow a quantitative description of fly trajectories (Kohlhoff et al, 2011). This assay could be utilised to see whether human GSK3β affects the motor neuron function and the climbing ability of aged flies expressing human tau. Finally, as firstly attempted in this study, mass spectrometry could be used to map each phosphorylated site produced by GSK3β on tau. This could help to clarify the role that specific phosphorylation sites play in generating tau toxicity during disease.

6.3 Mutation of S404 contributes to human tau toxicity in *Drosophila*

Phosphorylation of tau is highly complex with currently 45 identified phosphorylated residues and little information on the importance of each to tau toxicity (Hanger et al, 2007). The role of aberrant phosphorylation at SP/TP sites on human tau by *Drosophila* kinases has been proved to play a critical role in tau neurotoxicity in flies causing the protein to alter its physiological function and become toxic (Chatterjee et al, 2009; Chau et al, 2006; Jackson et al, 2002; Steinhilb et al, 2007b). The tau toxicity generated by endogenous kinases has been modulated using mutated forms of the tau gene that inhibit phosphorylation of single sites (Steinhilb et al, 2007a). These studies have shown that only the loss of all SP/TP phosphorylation sites on tau can block the toxicity produced by endogenous fly kinases. It has not been possible to identify the importance of individual phosphorylation sites to confer tau toxicity. Moreover, the transgenic *Drosophila* lines generated for this purpose have been using P-element mediated transgenesis. To overcome the variability of phenotype and of expression level of the transgene from P-element insertions, I created a model of tauopathy where the transgene insertion can be reproduced ensuring a standardised genetic background and expression level of the transgene. Using the phiC31 method, this thesis shows that S404 is an important site to mediate toxicity via human GSK3β in the fly eye. In particular, GSK3β

was able to generate greater eye degeneration when S404 was mutated compared to wild type tau. These results suggest that mutation of S404 increase tau toxicity possibly through inhibiting phosphorylation or through a conformational change that alters the ability of GSK3β to phosphorylate tau.

Based on these observations, I propose a model where a tight regulation of the phosphorylation and dephosphorylation events on S404 of human tau might be required to maintain the physiological function of tau. An increase in phosphorylation of S404 by human GSK3β was shown to correlate with an increase in tau-mediated toxicity. Indeed, increased phosphorylation of S404 has consistently been found in neurofibrillary tangles from AD brains and *in vitro* evidence shows that it is primarily responsible for the loss of tau-mediated tubulin polymerisation, suggesting a possible role in initiating the destabilisation of microtubules (Evans et al, 2000; Goedert et al, 1994; Kiris et al, 2011). This evidence supports the hypothesis that the detachment of human tau from microtubules due to phosphorylation by human GSK3β causes toxicity in the fly eye. At the same time, this thesis found that mutating S404 in a non-phosphorylatable form also increased the toxicity mediated by human GSK3β on wild type tau in flies. S404 dephosphorylation has been shown to result in the inhibition of anterograde transport *in vitro* (Tatebayashi et al, 2004). Moreover, in agreement with my study, mutation of S404 on human tau expressed in *Drosophila* has also been found to lead to increased toxicity upon shaggy expression. This increase toxicity could be due to a pathogenic detachment of human tau from microtubules which altered the regulation of their assembly leading to increased toxicity (Chatterjee et al, 2009).

Further experiments would be needed to investigate whether mutation of S404 in human tau leads to increased toxicity through conformational changes of tau or through toxic effects on the regulation of phosphorylation causing changes in the dynamics of microtubule assembly. The microtubule-binding activity of the mutated form of human tau could be studied *in vitro* on tau extracted from flies. The amount of tau bound to microtubules would be quantified by western blot as previously done (Chatterjee et al, 2009). Moreover, another approach could focus on investigating the consequences of the mutation on vesicle transport that relies on a strong network of microtubules. This could be performed in *Drosophila* as the translucent appearance of the larvae allows the transport of vesicles to be visualised in living animals. Using this technique, it would be

possible to see whether the mutation of the S404 residue affects the microtubule stability and therefore the vesicle transport in flies.

6.4 The N-terminal domains and the R406W mutation do not alter human tau-mediated degeneration in *Drosophila*

Tau exists in a number of isoforms and different mutated forms are associated with disease. Previous investigations studying human tau toxicity in *Drosophila* have used various tau isoforms and mutated forms of those. As explained above, the fly models of tauopathy established to date have been created using P-element mediated transgenesis. The use of different tau isoforms and the positional effect of the transgenes cause difficulties in the comparison between the different fly models. For this reason I exploited the phiC31-mediated transgenesis to generate *Drosophila* lines expressing different forms of the tau gene at the same level from the same genetic background. In contrast with previous studies, here I show that the FDTP-17 associated mutation R406W confers a similar level of toxicity as wild-type tau at low and high expression levels in the fly eye. Whereas, human 0N4R tau carrying the R406W mutation was demonstrated to be more toxic compared to wild type tau at higher expression levels in *Drosophila* (Nishimura et al, 2004; Wittmann et al, 2001). In previous work the positional effects caused by the random insertion of the R406W tau transgene in the fly genome are unknown, but they might affect the resulting phenotype. Using the site-specific method of insertion, the transgene expression level is lower and the genetic background of the fly lines is controlled. I found that the toxicity generated by the presence of none or two N-terminal domains was demonstrated to be very similar in fly lines generated using the phiC31-specific method of transgenesis. The fly models generated in this study suggest that the R406W mutation and the N-terminal domains do not appear to contribute to the tau-mediated degeneration in flies.

However, I found that the toxicity of the tau forms combined with the human kinases was shown to be different when tau was expressed at low and high levels in different genetic backgrounds. At low expression levels using the phiC31 lines, the kinase that confers greater toxicity to the all tau forms tested is human GSK3β. At higher

expression levels using the fly lines made with standard methods, many human kinases had a greater effect on the toxicity generated by 0N4R tau R406W rather then by the 2N4R isoform expressed at an equal level. The N-terminal domains and the R406W mutation do not contribute to generate tau-toxicity in flies when tau is expressed at low levels with or without human kinases. In contrast, at higher levels of expression, the absence of N-terminal domains and the presence of the R406W mutation on tau are more toxic than wild type tau containing two N-terminal domains in conferring toxicity with and without human kinases. It is possible that a considerably high level of transgene expression is needed in order for changes in toxicity to be identified. Once again these results show the importance of the expression level of the transgene the genetic background of the transgenic flies to be able to identify changes in toxicity of different tau isoforms.

As suggested above it would be useful to expand this study in an attempt to increase the transgene expression in flies created with the phiC31-mediated transgenesis. The introduction of insulators in the site of transgene insertion would possibly improve the system leading to a greater level of expression of tau and phenotype generated (Markstein et al, 2008).

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